TAXONOMIC CLASSIFICATION OF MYCOBACTERIA OF THE *MYCOBACTERIUM CHELONAE-MYCOBACTERIUM ABSCESSUS* COMPLEX WITHOUT CONCLUSIVE SPECIES IDENTIFICATION

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Dissertation submitted in fulfillment of the requirements for the degree of
Doctor (Ph.D.) in Sciences, Biochemistry and Biotechnology (Ghent University)
Doctor (Ph.D.) in Microbiology and Immunology (Universidade Federal de São Paulo)

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São Paulo, 24th October 2016

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ACKNOWLEDGEMENTS

Firstly, I would like to thank my advisor Prof. Sylvia Leão for the opportunity to carry out my PhD at UNIFESP, for the continuous support of my study, sharing with me her expertise, and for her sincere and valuable guidance. She definitely provided me the essential tools that I needed to complete my thesis.

I also would like to thank my labmate at UNIFESP Cristianne Matsumoto that always encouraged me with the experiments and helped me with her great wet-lab experience. To Vitória Dias, Mércia Vieira and all staff of Department of Microbiology, Immunology and Parasitology of UNIFESP for their various forms of supporting my study.

I would like to thank my advisor Prof. Peter Vandamme who provided me an unique opportunity to join his team doing a joint PhD at UGent, and who gave me access to all facilities of the Laboratory of Microbiology. I appreciate his technical support and assistance in thesis and papers writing.

In frame of my joint PhD I had gone to Ghent four times, where several people contributed to my research. I am really thankful to all Laboratory of Microbiology staff. My special thank to Anandi Martin who always warmly received me in Ghent; for her patience, motivation, expertise and friendy advices. To Juan Carlos Palomino for his insightful comments and expertise that greatly assisted my research. To Margo Cnockaert, for her parent-like support that made me feel at home during all my stay in Ghent; and of course for her support in DNA-DNA hibridization. To Cindy Snauwaert that kindly helped with her expertise in DNA extraction. And also to my labmates Joahana Monteresin, Marie Sylvianne and Beatriz Lopez for the happy time that we spent together during my stays at Ghent.

I would like to thank Prof. Françoise Portaels, Prof. Denise de Freitas, Prof. Christopher Whipps and Dr. Érica Chimara that kindly provided the isolates included in this study.

I am also thankful to Érica Chimara, Natália Fernandes, Romilda Aparecida Lemes and Fernanda Simeão from Instituto Adolfo Lutz, who helped me with their expertise in susceptibility testing and phenotypic analyses.

I would like to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for providing funds for this research (2012/13763-0).

Finally, I must express my gratitude to my family. A special thank to my lovely husband Lucas for supporting me spiritually through the process of researching and writing this thesis and to always encouraging me to do my best. To my parents Orlando and Margarida, my brother André and my sister Thais for providing me with unfailing support and continuous encouragement through my years of study. And to my adorable nephews, Luis Henrique and Benjamin, that fill my days with so much joy and happiness. This accomplishment would not have been possible without all of them.

THANK YOU!

HEEL HARTELIJK BEDANKT!

OBRIGADA!

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LIST OF ABBREVIATIONS

AFB	Acid-Fast Bacillus
ANI	Average Nucleotide Identity
BLAST	Basic Local Alignment Search Tool
bp	base pairs
CLSI	Clinical and Laboratory Standards Institute
DDH	DNA–DNA Hybridization
DNA	Deoxyribonucleic Acid
DST	Drug Susceptibility Testing
GGD	Genome-to-Genome Distance
HPLC	High-Performance Liquid Chromatography
hsp65	65-kDa heat shock protein
IJSEM	International Journal of Systematic and Evolutionary Microbiology
ITS	16S-23S Internal Transcribed Spacer
LB	Lysogeny Broth
MAC	*Mycobacterium avium* Complex
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization Time-Of-Light Mass Spectrometry
MIC	Minimum Inhibitory Concentration
MLSA	Multilocus Sequence Analysis
NaCl	Sodium Chloride
NGS	Next-Generation Sequencing
NTM	Nontuberculous Mycobacteria
TETRA	Tetranucleotide frequency correlation coefficients
PRA	PCR Restriction Enzyme Analysis
PCR	Polymerase Chain Reaction
PMF	Peptide Mass Fingerprint
rpoB	Gene encoding the β-subunit of the RNA polymerase
RGM	Rapidly Growing Mycobacteria
rRNA	Ribosomal RNA

SGM	Slowly Growing Mycobacteria
SNP	Single Nucleotide Polymorphisms
sp. nov.	Species nova
subsp.	subspecies
WGS	Whole Genome Sequencing

OUTLINE OF THE THESIS

PART I comprises the background of this thesis and the description of the main objectives of this study.

PART II consists of a comprehensive literature overview on *Mycobacterium* genus with special focus on methodologies for mycobacteria identification and for description of new species. CHAPTER 1 describes the general characteristics of mycobacteria and the evolution of the *Mycobacterium* genus taxonomy over the years. CHAPTER 2 describes general characteristics of nontuberculous mycobacteria including their habitats, the medically important species and the commonest human diseases caused by them. It focuses on the *M. chelonae-M. abscessus* complex including its serial taxonomic classification changes. CHAPTER 3 summarizes the currently available phenotypic and molecular methodologies for identification of mycobacteria. CHAPTER 4 describes the antimicrobial susceptibility testing for rapidly growing mycobacteria. Finally, CHAPTER 5 provides an overview of the polyphasic approach used for description of new species and the minimal standards for assignment of new species to the *Mycobacterium* genus.

PART III presents the experimental work performed during this thesis. CHAPTER 6 describes the isolates and type strains included in this study. CHAPTER 7 describes the preliminary identification of the isolates included in this study. CHAPTERS 8 and 9 includes the description of *Mycobacterium franklinii* and *Mycobacterium saopaulense*, respectively, as novel species of the *M. chelonae-M. abscessus* complex. CHAPTER 10 describes analysis of the remaining isolates by whole genome sequencing and the genome comparison of the isolates and type strain included in this study.

PART IV presents a general discussion of the results of this study. CHAPTER 11 includes a general summary justifying the performance of each part of this study. CHAPTER 12 includes some difficulties in species identification faced in each methodology performed in this study and the impact of this work.

PART V provides the conclusions of this study and future perspectives.

PART VI comprises an abstract of this thesis.

PART I: BACKGROUND AND OBJECTIVES

Background

The *Mycobacterium chelonae-Mycobacterium abscessus* complex comprises closely related rapidly growing mycobacteria that are responsible for a wide spectrum of opportunistic infections in humans, especially localized skin and soft tissue infections. The occurrence of nosocomial outbreaks caused by *M. chelonae-M. abscessus* complex has been increasingly reported.

This complex has undergone several taxonomic updates over the years. When this investigation started the species formally accepted were *M. chelonae*, *Mycobacterium immunogenum*, *Mycobacterium salmoniphilum*, *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii*. However the taxonomic relations of these species still generate several doubts, due to important similarities in their phenotypic and genotypic characteristics.

Commercial DNA strip assays for mycobacteria species identification, as INNO-LiPA® (Innogenetics, Belgium), were recently developed. In this assay, isolates of the *M. chelonae-M. abscessus* complex are subdivided into four genotypic clusters (CHI, CHII, CHIII and CHIV). During its development, a great variability in phenotypic tests of the CHII cluster isolates was observed (Prof. Françoise Portaels – personal communication). Moreover, when other genotypic methodologies were performed, they showed discordant results and the identification at species level was not achieved.

In previous projects of our laboratory we detected three isolates of the *M. chelonae-M. abscessus* complex that remained without conclusive identification at species level. Two of them were isolated from corneal specimens of patients with infectious crystalline keratopathy after LASIK (Laser-Assisted in Situ Keratomileusis) in 1999 and the last one was recovered from a lymph node biopsy in 2007. Genotypic analyses showed that these isolates were closely related to each other, to the members of *M. chelonae-M. abscessus* complex and to the JAN1 and JAN2 isolates. JAN1 and JAN2 were obtained from zebrafish and were classified as belonging to the *M. chelonae-M. abscessus* complex due to their phenotypic and genotypic characteristics. The performance of several phenotypic and genotypic methodologies showed that these isolates have overlapping characteristics with

several members of the *M. chelonae-M. abscessus* complex. Nonetheless, their definitive identification at species level was doubtful.

These five isolates and a set of isolates of the CHII INNO-LiPA® cluster, provided by Prof. Françoise Portaels, were submitted to a polyphasic approach in order to investigate their taxonomic position in the *M. chelonae-M. abcessus* complex.

This project is important because correct mycobacterial species identification is essential for epidemiological purposes, implementation of infection control measures and for therapeutic management, since some species present specific antimicrobial susceptibility patterns.

Objectives

General Objective

The main objective of the present study was to examine the taxonomic position of a set of isolates of *M. chelonae-M. abscessus* complex with inconclusive species classification.

Specific Objectives

In the first phase of this study, the isolates were preliminarily characterized using the commercial test Genotype® *Mycobacterium* CM and AS and the PCR Restriction Enzyme Analysis of the *hsp65* gene (PRA-*hsp65*) and 16S-23S ITS (PRA-ITS).

In the second phase of the project, phenotypic analyses traditionally used for the identification of mycobacteria, based on culture characteristics and biochemical features; drug susceptibility testing; and DNA sequencing of the 16S rRNA, *hsp65* and *rpoB* genes and the 16S-23S ITS were performed. Some isolates were subjected to DNA-DNA hybridization assay against the type strains of the *M. chelonae-M. abscessus* complex. With these data, two additional species of the *M. chelonae-M. abscessus* complex were fully described and validly named.

In the third phase of this study the isolates without conclusive taxonomic identification after the molecular identification and the *M. chelonae-M. abscessus* complex type strains were subjected to Whole Genome Sequencing (WGS). Several pairwise genome comparisons, including Average Nucleotide Identity (ANI), Tetranucleotide frequency correlation coefficient (TETRA) analysis, genomic signature (delta values), Genome-to-genome distance (GGD) and digital DNA-DNA hybridization, were performed.

PART II: LITERATURE

1 *Mycobacterium* sp

1.1 *Mycobacterium* sp – General characteristics

The *Mycobacterium* genus includes saprophytes, intermediate forms and obligate, facultative or opportunistic parasites. The majority of the species is widespread in the environment and can cause a wide range of diseases in both humans and animals. Diseases caused by mycobacteria differ in severity and have different impact in public health (Shamputa, Rigouts et al. 2004).

Mycobacteria are aerobic or microaerophilic, slightly curved and rod-shaped bacilli. They are non-motile and non-sporulating organisms and do not contain endospores or capsules. They have high G+C content (61-71 mol%), except for *Mycobacterium leprae* with 57.8 mol% G+C content, and a thick, hydrophobic and lipid-rich cell wall, mainly constituted by long-chain fatty acids containing 60-90 carbon atoms, the mycolic acids. Mycolic acids are responsible for the characteristic resistance to decolorization by acid-alcohol during the Ziehl-Neelsen staining procedure. Because of this, mycobacteria are known as Acid-Fast Bacilli (AFB) (Levy-Frebault and Portaels 1992, Rastogi, Legrand et al. 2001). Moreover, the thickness and composition of the cell wall render mycobacteria impermeable to hydrophilic nutrients and resistant to heavy metals, disinfectants, and antibiotics (Jarlier and Nikaido 1994).

Different species have different optimal growth temperatures within a range of 25°C-45°C. The generation time is usually long and varies widely in different species. Mycobacteria that form visible colonies to the naked eye within seven days on solid medium are named Rapidly Growing Mycobacteria (RGM), while those requiring more than 7 days are named Slowly Growing Mycobacteria (SGM) (Bergey, Harrison et al. 1923). This classification is supported by phylogenetic trees constructed with different housekeeping genes, in which RGM and SGM are clearly separated, forming distinct groups (Devulder, Perouse de Montclos et al. 2005, Tortoli 2012). A preliminary phylogenetic study using supertrees concluded that SGM evolved as a subgroup of RGM (Mignard and Flandrois 2008).

Some mycobacteria produce deep yellow to orange colonies when grown in light or darkness (schotochromogens). Others are non-pigmented in the light and dark (nonchromogens) and others produce non-pigmented colonies when grown in the dark and pigmented colonies after photoactivation (photochromogens) (Bergey, Harrison *et al*. 1923).

1.2 *Mycobacterium* sp – Taxonomy

The *Mycobacterium* genus is the single genus within family Mycobacteriaceae, order Actinomycetales. Taxonomically, it has undergone constant updates and the number of species has been increasing over the years (Rastogi, Legrand et al. 2001, Tortoli 2006).

The first species described of *Mycobacterium* genus was *M. leprae*, which was identified by Hansen in 1874 (Skerman, Mcgowan *et al*. 1980). Then, in 1882, Robert Koch isolated bacilli tubercle from infected mammalian tissue and proved that they were the causal agent of tuberculosis. Subsequently, *Mycobacterium tuberculosis* and *Mycobacterium bovis* were described as causative agents of human and bovine tuberculosis, respectively (Smith 1896, Karlson and Carr 1970, Karlson and Lessel 1970). Historically, for many years only *M. tuberculosis* and *M. leprae* were recognized as responsible for human infections (Moore and Frerichs 1953).

Therefore, until 1950s, it was very common to diagnose as tuberculosis all lesions in which AFB were detected. However, in the following years, it was demonstrated that, bacteriologically, mycobacteria isolated from some infections were very different from the bacilli of tuberculosis and leprosy. Subsequently, other species of mycobacteria began to be described (Moore and Frerichs 1953). These mycobacteria were previously known as anonymous, atypical mycobacteria and Mycobacteria Other Than Tuberculosis (MOTT). However, nowadays the universally accepted terminology is Nontuberculous Mycobacteria (NTM).

The increasing description of new NTM species prompted the necessity of suitable mycobacteria identification and classification methods. The first strategies employed to identify NTM species included bacilli staining characteristics, cultural morphology and biochemical tests. A proper classification system of mycobacteria started with Timpe and Runyon (Timpe and Runyon 1954). Subsequently, a

classification system based on growth rate and pigment production on solid medium was introduced by Runyon (Runyon 1958). According to this classification, mycobacteria were subdivided into four groups: I. Slow grower photochromogens; II. Slow grower scotochromogens; III. Slow grower nonchromogens; IV. Rapid growers.

In 1967 the “International Working Group on Mycobacterial Taxonomy” was formed and during the following 10 years this group worked on classification using numerical taxonomy within Runyon groups. In numerical taxonomy a number of phenotypic characteristics are used for grouping bacterial strains based on statistical significance of the obtained results. These extensive studies provided a phenotypical identification scheme for NTM species (Wayne, Dietz et al. 1971, Kubica, Baess et al. 1972, Meissner, Schroder et al. 1974, Saito, Gordon et al. 1977, Tsukamura and Mizuno 1977, Wayne, Andrade et al. 1978).

During this period just over 40 species were known and the recommended tests appeared suitable for identification of the clinically important species of NTM. However, because their low discrimination ability ambiguities in species identification emerged, leading to diverging opinions concerning the classification of mycobacteria. Moreover, phenotypic tests usually assigned isolates to the species they best resemble among those previously described leading to misidentifications (Butler and Guthertz 2001, Tortoli, Bartoloni et al. 2001). According to Tortoli *et al*. this was the reason why mycobacterial taxonomy remained unchanged during several years (Tortoli, Bartoloni et al. 2001).

In the late 1970s/early 1980s other methodologies began to be used in order to clarify questions regarding mycobacterial taxonomy. The determination of DNA-DNA Hybridization (DDH) percentages and the use of High-Performance Liquid Chromatography (HPLC) analysis of the mycolic acids became popular. The use of these methodologies led to different conclusions and several mycobacteria classifications were revised (Baess and Bentzon 1978, Butler and Guthertz 2001). Later, DNA sequencing of 16S rRNA and other housekeeping genes were also introduced, contributing for the delineation of mycobacteria species (Telenti, Marchesi et al. 1993, Lappayawichit, Rienthong et al. 1996, Kirschner and Bottger 1998, Park, Jang et al. 2000, Adekambi, Berger et al. 2006).

The introduction of these methodologies provided a continuous evolution of mycobacteria taxonomy. It led to an exponential increase in the number of

descriptions of new mycobacteria species over the last decades (Tortoli 2006, Dai, Chen et al. 2011) (Figure 1.1). However, even with the availability of several molecular methodologies, there are still diverging opinions concerning the classification of mycobacteria.

Currently the *Mycobacterium* genus comprises more than 170 species (J. P. Euzéby, List of Prokaryotic Names with Standing in Nomenclature [http://www.bacterio.citc.fr/m/mycobacterium.html]) including the etiological agents of tuberculosis (*M. tuberculosis* complex) (Gagneux 2012), leprosy (*M. leprae*) and the large group of NTM (Dawson 2000).

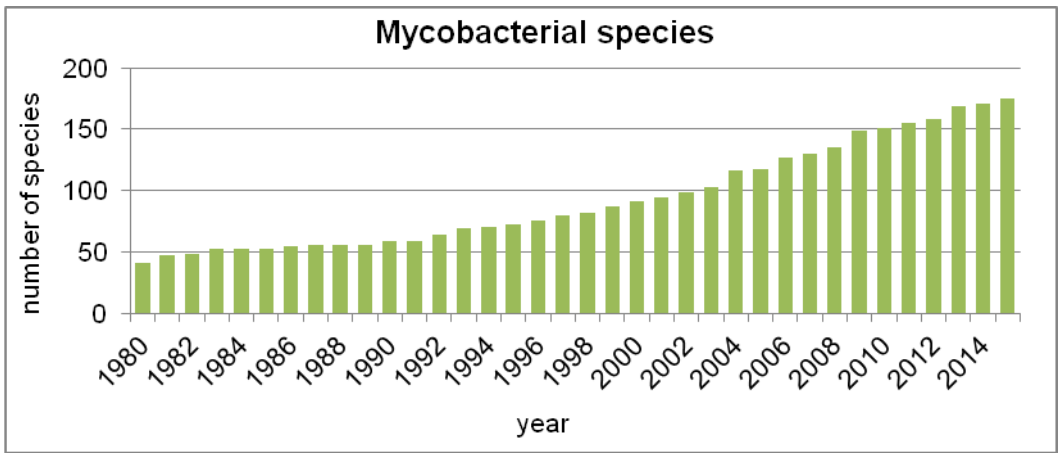

Figure 1.1. Numbers of officially accepted *Mycobacterium* species from 1980 to 2015 in the List of Prokaryotic names with Standing in Nomenclature. This graphic was built based on the List of Prokaryotic Names with Standing in Nomenclature [http://www.bacterio.citc.fr/m/mycobacterium.html.

2 Nontuberculous Mycobacteria (NTM)

NTM is a heterogenic group that encompasses the majority of species in the *Mycobacterium* genus. Unlike *M. tuberculosis* complex and *M. leprae*, NTM are not obligate pathogens but are free living bacteria that are spread in the environment. They can be found as saprophytes, commensals and symbionts in soil and ground water, in sewage and dust as well as in fresh and sea water (Primm, Lucero et al. 2004). Therefore NTM are inhabitants of several environmental habitats that are shared with animals and humans, including drinking water distribution systems, household plumbing and water systems in hospitals, hemodialysis centers, surgical

facilities, dental offices (Phillips and von Reyn 2001, Falkinham 2002, Falkinham 2009, Falkinham 2013) (Table 2.1).

Table 2.1. Environmental isolations of mycobacteria. Adapted from (Primm, Lucero et al. 2004)

NTM Species	Location
M. ulcerans	Natural waters, soil, insects, wild animals, fish
M. scrofulaceum, MAC, *M. szulgai*, *M. fortuitum*	Surface water
M. gordonae, MAC	City aquarium, drinking water
MAC	Residential water sources (i.e. toilet, tap, shower)
MAC	Hospital recirculating hot water system
M. mucogenicum, *M. kansasii*, *M. gordonae*, MAC, *M. fortuitum*	Public drinking and potable water sources, ice machines, water treatment plant
MAC, *M. gordonae*, *M. fortuitum*, *M. kansasii*, *M. marinum*, *M. chelonae*	Public swimming pools and whirlpools
MAC	Hot tubs
M. scrofulaceum, MAC, *M. szulgai*, *M. fortuitum*, *M. gordonae*, *M. simiae*	Hospital tap water
M. fortuitum, MAC	Soil
M. fortuitum	Sewage treatment plant
M. flavescens, *M. austroafricanum*, *M. chlorophenolicum* and unknown*	Petroleum-contaminated soil
Many species	Domestic and wild animals, numerous species
M. immunogenum	Biocide-treated metalworking fluid
M. chelonae	Gentian violet solution

*Unknown, 16S ribosomal sequence did not match any known species.
MAC, *M. avium* Complex.

There has been an increasing awareness of a variety of opportunistic diseases caused by NTM, which were named mycobacterioses (Johnson and Odell 2014, Mwikuma, Kwenda et al. 2015). Being classical opportunists, NTM predominantly infect patients already suffering from pulmonary diseases or immunodeficiency; however, the number of mycobacterioses is also increasing among immunocompetent persons (Primm, Lucero et al. 2004).

NTM person-to-person transmission has not been demonstrated. Environmental exposure coupled with several inherent characteristics of NTM contributes for the occurrence of NTM opportunistic infections by aerosolization and inhalation, ingestion, or dermal contact with contaminated sources (Primm, Lucero et al. 2004, Donohue, Mistry et al. 2015). Lymphadenitis, pulmonary and skin and soft tissue infections are the most commonly described human infections (Johnson and Odell 2014).

The hydrophobicity of mycobacteria results in preferential aerosolization from water during daily activities such as drinking, swimming and bathing leading to recurrent human exposure (Parker, Ford et al. 1983, Falkinham 2002, Primm, Lucero

et al. 2004, Feazel, Baumgartner et al. 2009). This is the likely route of NTM pulmonary infection in immunocompromised patients. *Mycobacterium avium, Mycobacterium kansasii,* and *M. abscessus* are the most frequently identified organisms causing lung disease (Johnson and Odell 2014).

Immunocompromised patients are also affected by NTM lymphadenitis, however it is the most common NTM manifestation in young children. Pharyngeal mucosa, tonsils, conjunctiva, gingiva, and salivary glands are considered the NTM ports of infection, for example, during ingestion of contaminated soil or water. *M. avium* and *Mycobacterium haemophilum* are the most common cause of NTM lymphadenitis (Primm, Lucero et al. 2004, Amir 2010).

Mycobacteria hydrophobicity has also been associated with biofilm formation (Schulze-Robbecke, Janning et al. 1992), contributing to disinfectant and antibiotic resistance (Jarlier and Nikaido 1994, Falkinham 2007). The presence of NTM in water associated with disinfectant resistance and consequent failure in sterilization, leads to NTM presence in solutions used in medical interventions and in medical instruments and equipments (Thomson, Carter et al. 2013, Mwikuma, Kwenda et al. 2015). These factors associated with the increasing number of invasive (medical or non-medical) procedures, especially eye surgeries, laparoscopic procedures, plastic surgeries and cosmetic techniques (such as mesotherapy) have been identified as the main factors that generate NTM skin and soft tissue infections. The breaking down of the integrity of skin, corneal, or mucosal barriers leads to NTM inoculation and to the development of mycobacterial diseases and nosocomial outbreaks (Martinez, McAdams et al. 2007). During the last decades there has been a significant increase in post-traumatic and post-operative infections due to these organisms. Nearly every species of NTM may cause skin and soft tissue infections, however RGM are the most commonly involved, specially the members of *M. chelonae-M. abscessus, Mycobacterium fortuitum* and *Mycobacterium smegmatis* complexes (Griffith, Aksamit et al. 2007, Alcaide and Esteban 2010).

2.1 *M. chelonae-M. abscessus* complex

The *M. chelonae-M. abscessus* complex comprises closely related RGM that can cause several opportunistic infections in both immunocompromised and in

immunocompetent persons. They have been mainly involved in pulmonary, skin and soft tissue infections and disseminated disease (especially in immunocompromised patients) (Brown-Elliott and Wallace 2002, Falkinham 2013, Nunes Lde, Baethgen et al. 2014). A predisponsing factor frequently associated with pulmonary disease is the presence of cystic fibrosis (De Groote and Huitt 2006).

This complex has been increasingly involved in both community and hospital-acquired infections after trauma or surgery in immunocompetent persons, representing a serious public health problem (Koh, Jeon et al. 2011). Recent publications have indicated an increasing number of infections by *M. chelonae-M. abscessus* complex members associated with eye infections after laser in situ keratomileusis (LASIK), endocarditis, surgical wounds, post-injection, catheter and hemodialysis-related infections, infections after tattoos, as well as disseminated infections in immunosuppressed patients (Meyers, Brown-Elliott et al. 2002, Hansen and Sarma 2006, Sampaio, Junior et al. 2006, Del-Castillo, Palmero et al. 2009, Al-Benwan, Ahmad et al. 2010, Shedd, Edhegard et al. 2010, Mitchell, Isenstein et al. 2011, Stout, Gadkowski et al. 2011, Wongkitisophon, Rattanakaemakorn et al. 2011, Kennedy, Bedard et al. 2012, D'Ancona, Kanitz et al. 2014, Bhandari, Sriganesh et al. 2015, Edens, Liebich et al. 2015, Henkle and Winthrop 2015, Jung, Kim et al. 2015, Shah, Relhan et al. 2016). Between 2004 and 2008, an epidemic of localized skin and soft tissue infections caused by members of this complex affecting more than 2,000 patients occurred in Brazil, following invasive procedures such as laparoscopic, arthroscopic and plastic surgeries, and cosmetic interventions (Cardoso, Martins de Sousa et al. 2008, Viana-Niero, Lima et al. 2008, Duarte, Lourenco et al. 2009, Leao, Viana-Niero et al. 2010, Nunes Lde, Baethgen et al. 2014).

2.1.1 Taxonomy of *M. chelonae-M. abscessus* complex

Similar to what occurred with the *Mycobacterium* genus as a whole, several changes in the taxonomic classification of the members of *M. chelonae-M. abscessus* complex occurred over the years.

Mycobacterium chelonei was described by Bergey *et al.* to include Friedmann's (1903) turtle tubercle bacillus. The etymology of *M. chelonei* refers to

Gr. n. *khelone*, pertaining to tortoise (any of various terrestrial turtles) (Bergey, Harrison et al. 1923).

Moore and Frerichs reported a human knee infection caused by mycobacteria probably after injections of streptomycin. Based on its phenotypical characteristics, a new species was described and named *M. abscessus* – L. gen. n. *abscessus*, of an abscess, referring to its ability to produce deep abscesses in human tissue (Moore and Frerichs 1953).

Based on bacteriological and serological examinations, Stanford and Beck (Stanford and Beck 1969) found that the following strains were similar to the turtle tubercle bacillus: *M. abscessus* (Moore and Frerichs 1953), *Mycobacterium runyonii* (Bojalil, Cerbon et al. 1962) and *Mycobacterium borstelense* (Bonicke 1965). Therefore they were considered synonyms of *M. chelonei*. Subsequently, two subgroups of *M. chelonei* were recognized and distinguished by several biochemical tests included in the numerical taxonomy. Consequently, they were classified as *M. chelonei* subsp. *chelonei* and *M. chelonei* subsp. *abscessus* (Kubica, Baess et al. 1972, Stanford, Pattyn et al. 1972). All other synonyms previously described for this species were considered invalid (Ward 1975).

For many years the cause of chronic abscesses after injury or injection was attributed to *M. chelonei* and *M. fortuitum*. *M. fortuitum* was isolated from a post-injection abscess in a Brazilian patient by da Costa Cruz and *M. peregrinum* was first proposed as a RGM species distinct from *M. fortuitum* (Da Costa Cruz 1938, Bojalil, Cerbon et al. 1962). Later, Stanford and Gunthorpe demonstrated that these two taxa belong to a single species based on serological tests (Stanford and Gunthorpe 1969). Therefore, *M. peregrinum* was considered synonym of *M. fortuitum* (Kubica, Baess et al. 1972).

Strains of *M. chelonei* subsp. *chelonei*, *M. chelonei* subsp. *abscessus* and *M. fortuitum* (*M. peregrinum*) were distinguished by some biochemical and colonial properties (Pattyn, Magnusson et al. 1974) and in vitro antibiotic sensibility studies (Haas, Michel et al. 1973). However, even with phenotypic characterization and numerical taxonomy, the differentiation of *M. chelonei* subsp. *chelonei*, *M. chelonei* subsp. *abscessus* and *M. fortuitum* remained difficult and different opinions concerning their species status emerged (Baess 1982). In consequence, based on

numerical analysis, the three taxa were reunited in the *M. fortuitum* complex (Tsukamura 1981).

In the following years, Hill *et al*. corrected the name *M. chelonei* to *M. chelonae*, given the gender of the turtle name, *Chelona corticata* (Hill, Skerman et al. 1984).

In the 1980s, when the performance of DDH experiments became common, important taxonomic changes occurred in the *M. fortuitum* complex. Baess was the first author to demonstrate the low DNA relatedness between both subspecies of *M. chelonae* and *M. fortuitum* (*M. peregrinum)* (Baess 1982). These findings were complemented by Levy-Frebault *et al*., who clearly demonstrated that *M. chelonae* subsp *chelonae* and *M. chelonae* subsp. *abscessus* were independent species and both differed from *M. fortuitum* and *M. peregrinum* (Levy-Frebault, Grimont et al. 1986). In 1992 Kusunoki and Ezaki confirmed the previous findings using different conditions of DDH experiments leading to the elevation of *M. chelonae* subsp. *abscessus* to the species status of *M. abscessus* and the revival of *M. peregrinum* as an independent species (Kusunoki and Ezaki 1992). Moreover, *M. chelonae* and *M. abscessus* were further grouped in the *M. chelonae-M. abscessus* complex.

Besides DDH experiments, several molecular methodologies including DNA sequencing and HPLC analysis of the mycolic acid were introduced. Consequently, it became clear that both *M. chelonae* and *M. abscessus* were more closely related to each other than to *M. fortuitum.* Futhermore, using a polyphasic approach, new species closely related to both *M. chelonae* and *M. abscessus* were described and diverging opinions concerning their taxonomy emerged (Wilson, Steingrube et al. 2001, Adekambi, Berger et al. 2006, Whipps, Butler et al. 2007, Leao, Tortoli et al. 2011).

In 2001, Wilson *et al*. studied a set of isolates of non-pigmented RGM from clinical and environmental sources that failed to match with patterns of officially accepted species. They showed phenotypical and HPLC profiles compatible with *M. abscessus* and *M. chelonae*. However, 16S rRNA gene sequencing and DDH clearly demonstrated that they belonged to a new species of the *M. chelonae-M. abscessus* complex. The name *Mycobacterium immunogenum* sp. nov. was proposed – N.L. neut. adj. *immunogenum*, refering an immune response – and validly published (Wilson, Steingrube et al. 2001).

In 2006, Adékambi *et al*. performed a polyphasic approach study in a collection of clinical RGM isolates recovered from bronchial and stomach aspirates, sputum and joint fluid (Adekambi, Berger et al. 2006). A new member of the *M. chelonae-M. abscessus* complex was validly published, *Mycobacterium bolletii* sp. nov. – N.L. gen. masc. n. *bolletii*, of Bollet, to honour Claude Bollet, a famous clinical microbiologist and taxonomist .

In the same year, *Mycobacterium massiliense* was officially accepted as a new member of *M. chelonae-M. abscessus* complex (Adekambi, Reynaud-Gaubert et al. 2004a). It was isolated from sputum and bronchoalveolar fluid of a patient with hemoptoic pneumonia. Phenotypic and genotypic features of the isolates indicated that they were representative of a new species, *M. massiliense* – L. neut. adj. *massiliense*, refering to Massilia, the Latin name of Marseille, where the organism was firstly isolated.

In 2007, Whipps *et al*. employed a polyphasic approach in order to clarify the taxonomic position of mycobacteria isolated from salmon (Whipps, Butler et al. 2007). A *M. chelonae*-like species from salmon, '*Mycobacterium salmoniphilum'*, was described in 1960 (Ross 1960) – L. n. salmo -onis, a salmon; N.L. adj. philus -a -um (from Gr. adj. philos -ê -on), friend, loving; N.L. neut. adj. salmoniphilum, salmon-loving. However, because it could not be distinguished from *M. fortuitum*, *M. salmoniphilum* was omitted from the 1980 Approved Lists of Bacterial Names. In the 1980s, the mycobacteria isolated from salmon were characterized as a subspecies of *M. chelonae*, named '*M. chelonae* subsp. *piscarium*'. However, there were still doubts about the existence of this subspecies and, therefore, it was subsequently removed from the list of valid names. Again, in 2007, Whipps *et al*. examined the taxonomic relatedness of type strains and authentic isolates from earlier studies. The results supported the status as a separate taxon and *M. salmoniphilum* was officially accepted (Whipps, Butler et al. 2007).

Leão *et al*. (Leao, Tortoli et al. 2009, Leao, Viana-Niero et al. 2010) studied isolates of an outbreak related to laparoscopic and arthroscopic surgeries. This outbreak started in Belém (Pará, Brazil) and then spread to different Brazilian geographic areas. This study showed that the outbreak was related to a single strain, which was initially identified as *M. massiliense* (Cardoso, Martins de Sousa et al. 2008, Viana-Niero, Lima et al. 2008, Duarte, Lourenco et al. 2009, Leao, Tortoli et al.

2009). In fact, the identification results were controversial because of the high level of similarity of *rpoB* and *hsp65* gene sequences with the corresponding sequences of both *M. massiliense* and *M. bolletii* (Viana-Niero, Lima et al. 2008, Leao, Tortoli et al. 2009). In order to confirm the species identification, the isolates from this outbreak and the type strains of *M.chelonae-M. abscessus* complex were extensively studied using a polyphasic approach. The results showed that these isolates could not be clearly discriminated by phenotypic or molecular tests, including the gold standard DDH. Therefore, *M. abscessus, M. massiliense* and *M. bolletii* were reunited in a single species – *M. abscessus* – with two subspecies – *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* (Leao, Tortoli et al. 2009, Leao, Tortoli et al. 2011). Subspecies *bolletii* included those isolates identified previously as *M. massiliense* or *M. bolletii*. The name *M. abscessus* subsp. *bolletii* was chosen on the basis of the Bacteriological Code (1990 Revision) (Lapage, Sneath et al. 1992, De Vos and Trüper 2000). Since the name *M. bolletii* was validly published on January/2006 and the *M. massiliense* name on September/2006, the name *M. bolletii* had priority over the name *M. massiliense*. This reclassification was officially accepted in 2011 (Leao, Tortoli et al. 2011).

Recently, a debate has ensued over the reclassification of *M. massiliense* and *M. bolletii* in a single subspecies - *M. abscessus* subsp. *bolletii*. Several recent studies based on genome comparisons have provided evidence that there can indeed be three taxonomic groups within *M. abscessus*: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii* (Bryant, Grogono et al. 2013, Heydari, Wee et al. 2013, Benwill and Wallace 2014, Sassi and Drancourt 2014, Tettelin, Davidson et al. 2014). Whole Genomic Sequencing and Single Nucleotide Polymorphisms (SNP) information contained in the core genomes were used to construct phylogenetic trees that show deep genetic divisions corresponding to three different taxa. It is important to mention that the number of isolates identified as *M. bolletii* or *M. abscessus* subsp. *bolletii* in these studies is extremely low (1-3) in comparison to the number of isolates identified as *M. abscessus* (*M. abscessus* subsp. *abscessus*) or *M. massiliense* (*M. abscessus* subsp. *massiliense*). This can generate bias to the conclusion that *M. massiliense* and *M. bolletii* are separate taxa. Moreover, there are several reports describing isolates of *M. bolletii* and *M. massiliense* that cannot be clearly classified by available

molecular methods (Kim, Kook et al. 2008, Viana-Niero, Lima et al. 2008, Zelazny, Root et al. 2009). However, it is not clear yet how this novel information will affect the 2011 reclassification.

Other species were proposed as new members of the *M. chelonae-M. abscessus* complex, but their names were not validly published. In 2011, Simmon *et al*. studied a group of clinical isolates from Pennsylvania and Northeastern United States that wrongly assigned as *M. chelonae* with an atypical antimicrobial susceptibility pattern (Simmon, Brown-Elliott et al. 2011). This study included the proposal of '*Mycobacterium franklinii'* as a new member of *M. chelonae-M. abscessus* complex. Zhang *et al*. investigated clinical isolates previously identified as belonging to *M. chelonae-M. abscessus* complex (Zhang, Li et al. 2013). Their *hsp65* sequences showed more than 99% identity with the respective sequences of *M. abscessus*, however, low identity was verified in *rpoB*, ITS, *sodA*, and *recA* sequences. Therefore, a new species was proposed, '*Mycobacterium fukienense'*.

Currently the taxonomic relations of members of the *M. chelonae-M. abscessus* complex still generate several doubts due to important similarities in phenotypic and genotypic characteristics hindering their identification at the species level. Species are biochemically inert and partial sequences of the 16S rRNA gene are too similar, making their identification a challenge. Nonetheless, definitive identification by the clinical laboratory is extremely important for outbreak detection and for patient management (Simmon, Brown-Elliott et al. 2011).

3 Mycobacteria Identification Methods

The NTM species are genetically closely related to each other making their identification at species level a challenge especially for clinical laboratories (Tortoli 2003). However, the rapid identification at the species level is essential for several reasons. First of all, NTM species identification is needed to define the value of an isolate as a true pathogen or an environmental and potential contaminant (Leão, Martin et al. 2004). Moreover, it is important to discriminate the NTM species for therapeutic management since some species present characteristic antimicrobial patterns (Tortoli 2003). Species identification is also important for epidemiological purposes and consequently for implementation of infection control measures.

Different laboratory techniques are available for mycobacteria species identification, including conventional methods based on phenotypic characteristics and biochemical tests, mycolic acid analysis and molecular methods.

3.1 Phenotypic Methods

The phenotypic identification of mycobacteria is based on characteristics of the culture and biochemical features, and comprises a large variety of tests. They were extensively used for identification and classification of mycobacteria mainly in the era of numerical taxonomy (Butler and Guthertz 2001).

However, as the number of species increased, these tests were shown to be unable to distinguish between all recognized mycobacteria species. Interspecies homogeneity, intraspecies variability, and the existence of species not yet described can lead to erroneous identification. For many NTM species, the phenotypic tests are poorly reproducible and for those biochemically unreactive, such as the members of *M. chelonae-M. abscessus* complex, they are really not useful for species discrimination. Furthermore, they are time-consuming and cause delay in the final species identification since the slow growth rate of mycobacteria. They are limited by subjectivity, low specificity and require experience in results interpretation (Butler and Guthertz 2001, Tortoli, Bartoloni et al. 2001, Cook, Turenne et al. 2003, Chimara, Ferrazoli et al. 2008, Simmon, Brown-Elliott et al. 2011).

Because of all these disadvantages, only few phenotypic tests described for NTM identification are still used in clinical laboratories. In general, first of all a smear of the culture is stained by Ziehl-Neelsen (or other acid fast stain) to confirm the presence of AFB. Then, the colony morphology (smooth or rough), growth rate (SGM or RGM), growth temperature (25^{o}C and 37^{o}C), and pigment production are examined. Moreover, the addition of specific growth inhibitors as para-nitrobenzoic acid (PNB) is used for the differentiation of NTM as a group because it inhibits only species belonging to the *M. tuberculosis* complex (Tsukamura 1984, Kent and Kubica 1985, Leão, Martin et al. 2004). For the final identification, other methods are employed, especially molecular tests.

3.2 Mycolic acid analysis

Another phenotypic characteristic that is used for mycobacteria identification is the mycolic acid pattern that can be determined using High-Performance Liquid Chromatography (HPLC) (Tortoli and Bartoloni 1996).

HPLC analysis is based on the fact that mycobacterial cell wall mycolic acids differ in the number of carbon atoms, ranging from 60 to 90, and in the presence of different functional groups. Therefore, according to the polarity and carbon chain length, the mycolic acids are separated and a HPLC pattern is generated. Then several characteristics are analyzed such as number, position, and height of peaks, which are useful for differentiate species of mycobacteria (Tortoli 2003).

The advantages of mycolic acid analysis are that it is rapid, not expensive and can identify several species and reveal unknown species (Tortoli, Bartoloni et al. 2001, Leão, Martin et al. 2004). Although its performance is more rapid than conventional biochemical tests, HPLC also requires initial culture of isolates on solid medium delaying the species identification and consequently the initiation of an efficient antimicrobial therapy. In addition, it has been shown that HPLC is inadequate to differentiate between closely related species such as some members of *M. chelonae-M. abscessus*, *M. avium* and *Mycobacterium mucogenicum* complexes (Griffith, Aksamit et al. 2007, Adekambi 2009, Koh, Jeong et al. 2012, van Ingen 2013).

3.3 Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS)

In recent years, microbiology laboratories are using Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) for bacterial identification from culture and directly from clinical samples. Proteins, mainly represented by ribosomal proteins, are extracted and mixed with an organic matrix solution that enables protein ionization. The ionized proteins are subsequently separated based on their mass/charge ratio producing a spectrum named Peptide Mass Fingerprint (PMF). As PMF is, in general, unique for a particular microorganism, the PMF pattern of an unknown isolate can be compared with

patterns deposited in databases to define its identification (Singhal, Kumar et al. 2015). Commercial MALDI-TOF MS systems with PMF patterns database are available, as MALDI BioTyper (Bruker Daltonics, Germany) and VITEK MS RUO (Research Use Only, bioMérieux, France) (Welker 2011, DeMarco and Ford 2013).

Studies have shown that MALDI-TOF MS is a rapid, accurate and reliable methodology for identification of medically important bacteria (Seng, Drancourt et al. 2009, Bizzini, Durussel et al. 2010, Benagli, Rossi et al. 2011, Neville, Lecordier et al. 2011). For mycobacteria identification, some studies have shown that MALDI-TOF MS correctly identified >90% of isolates recovered from solid medium (Pignone, Greth et al. 2006, Lotz, Ferroni et al. 2010, El Khechine, Couderc et al. 2011, Saleeb, Drake et al. 2011). However, for correct species identification these studies needed to build their own reference libraries or to complement the available commercial databases by addition of PMF patterns of reference strains. Moreover, several challenges have been faced to achieve reliable identification rates. For safety reasons, mycobacteria need to be inactivated; moreover, cell wall lysis protocols used for other microorganisms are inadequate to lyse the complex mycobacteria cell wall structure. Therefore, in these studies different protocols available for inactivation and cell lysis were tested or a particular protocol was developed. The use of different protocols could lead to differences in protein extraction efficiency and consequently in the generated PMF patterns, what could be a problem when using the available commercial databases.

Furthermore, important differences in accuracy and identification rates were reported with NTM isolates recovered from solid or liquid medium. Buchan *et al*. showed best results in identification when mycobacteria were analyzed directly from liquid culture; while Lotz *et al*. showed higher performance when isolates were analyzed directly from solid culture (Lotz, Ferroni et al. 2010, Buchan, Riebe et al. 2014). Recently, van Eck *et al*. showed low percentage in NTM identification when using isolates directly from liquid cultures of respiratory samples; a subculture on solid medium was required to obtain good quality identification results (van Eck, Faro et al. 2016).

Therefore, the lack of well standardized protocols for mycobacteria inactivation and protein extraction, the necessity to build new database and differences in the quality of identification when mycobacteria are analyzed from liquid or solid cultures

hinder MALDI-TOF MS implementation in clinical settings as a routine mycobacteria identification method.

3.4 Genotypic Methods

To meet the necessity of more rapid and accurate mycobacteria identification at species level, several molecular assays have been developed and introduced in clinical mycobacteriology laboratories. These assays are rapid and offer high sensitivities and specificities, especially when using DNA from mycobacterial culture (Tortoli 2003).

In general, they are based on the detection of highly conserved regions within the genome that harbor hypervariable sequences in which species-specific deletions, insertions, or single nucleotide polymorphisms are present (Leão, Martin et al. 2004). DNA sequencing and Polymerase Chain Reaction Restriction Enzyme Analysis (PRA), as well DNA strip assays are among the genotypic methods currently used for mycobacteria identification.

3.4.1 DNA Sequencing

Advances in molecular sequencing of the conserved genes 16S rRNA, *rpoB*, and *hsp65* and the 16S-23S ITS enabled faster NTM species identification at species level. The widespread use of these methodologies led to the discovery of new species and the taxonomic reclassification of others. As a consequence the number of NTM species officially accepted increased (Joao, Cristovao et al. 2014). Moreover, these methods facilitate comparisons of identification between different laboratories since they allow public database construction (Devulder, Perouse de Montclos et al. 2005).

3.4.1.1 16S rRNA

The 16S rRNA gene has about 1,500 nucleotides in length. It is universally used for bacterial species identification since it has hypervariable regions that allow discrimination of many species but often lacks resolution to distinguish closely related species. These regions have high variability between species but relatively low variability within species (Tortoli 2003, Leão, Martin et al. 2004). For mycobacterial species identification, the sequencing of an initial region is generally sufficient. It corresponds to positions 130 to 210 in the *Escherichia coli* 16S rRNA gene (Tortoli 2003).

Today, the 98.65% is used as a threshold as determined by Kim *et al*. (Kim, Oh et al. 2014). In spite of this, the restriction known for many years is still valid: organisms that differ more than this threshold value generally represent different species but organisms that differ less than 1.35% in their full 16S rRNA gene sequences may or may not belong to a single species.

The 16S rRNA molecule simply lacks resolution to distinguish among such closely related species due to little variations in their sequences, such as the following closely related NTM species: *M. abscessus* and *M. chelonae*; *M. avium* and *Mycobacterium intracellulare*; *M. peregrinum* and *Mycobacterium septicum*; *M. kansasii* and *Mycobacterium gastri*; *Mycobacterium marinum* and *Mycobacterium ulcerans; Mycobacterium houstonense* and *Mycobacterium senegalense* (Rogall, Wolters et al. 1990, Adekambi, Colson et al. 2003, Adekambi and Drancourt 2004b, Joao, Cristovao et al. 2014).

Although rare, the presence of two different copies of 16S rRNA in the same microorganism represents another limitation of its use for taxonomic purposes. In *Mycobacterium* genus, RGM have two copies of 16S rRNA, except *M. chelonae* and *M. abscessus* (Tortoli 2003). Different 16S rRNA sequences in the same mycobacterium were identified in the *Mycobacterium terrae* and *Mycobacterium celatum* complexes (Ninet, Monod et al. 1996, Reischl, Feldmann et al. 1998).

3.4.1.2 *rpoB*

rpoB is a single-copy gene that encodes the β-subunit of RNA polymerase responsible for the enzyme catalytic function (Adekambi, Colson et al. 2003). Since *rpoB* sequences of most clinically important species are available in public databases, this gene is considered a reasonable first approach for NTM isolated from clinical samples (de Zwaan, van Ingen et al. 2014).

Five variable regions of 420 to 780 bp length were identified in *rpoB* sequences of RGM, which have >5% mean variability. The hypervariable region that extends from position 2581 to 3300 of the *M. tuberculosis* gene was identified as the most suitable for identification of RGM at the species and subspecies levels (Adekambi, Colson et al. 2003). This region was named region V and it has been widely used for RGM identification as well in taxonomic studies (Adekambi and Drancourt 2004b, Adekambi, Berger et al. 2006, Whipps, Butler et al. 2007, Tortoli 2009).

Nevertheless some pitfalls in the use of *rpoB* gene for taxonomic purposes have been identified. The intraspecies degree of *rpoB* sequence similarity was estimated to range from 98.2 to 100% (Adekambi, Shinnick et al. 2008). However, high internal variability was detected within some members of the *M. abscessus-M. chelonae* complex, which represents a challenge in species identification. (Adekambi, Colson et al. 2003) showed that *M. abscessus* isolates have >4.3% sequence divergence in region V of the *rpoB* gene.

Another limitation of the *rpoB* gene is the occurrence of horizontal transfer between bacterial isolates as was for example demonstrated between *M. abscessus* isolates. Therefore its use as a single approach for NTM identification can be a pitfall (Macheras, Roux et al. 2011).

3.4.1.3 16S-23S Internal Transcribed Spacer (ITS)

The ITS between the 16S rRNA and 23S rRNA genes is more variable than the 16S rRNA gene and it is species specific in several bacteria (Park, Jang et al. 2000). Because of the high level of ITS sequence variation and the good reproducibility, it is considered a suitable target for differentiating clinically relevant

mycobacterial species (Lappayawichit, Rienthong et al. 1996, Park, Jang et al. 2000). The only mycobacterial species that have identical ITS sequences are the members of the *M. tuberculosis* complex (Frothingham, Hills et al. 1994).

In the *Mycobacterium* genus the ITS sequences length varies among the species, but in general it has 270 to 360 bp (Roth, Reischl et al. 2000). It has several polymorphic regions and only two conserved regions. Since the two conserved regions are near to each other, primers could be designed and the hypervariable region between them amplified. The size of this amplified region is variable. In general, the ITS sequences length of RGM are 75 nucleotides longer than those of SGM (Park, Jang et al. 2000).

Unfortunately some drawbacks have been identified in the use of ITS for taxonomic purposes. It failed to discriminate some closely related species such as *M. marinum* and *M. ulcerans*. Furthermore, the analysis of RGM ITS can be doubtful since the majority of these species have multiple rRNA operons and interoperon heterogeneities can occur (Roth, Fischer et al. 1998).

3.4.1.4 *hsp65*

The 65-kDa heat-shock protein (*hsp65*) gene is highly conserved in the *Mycobacterium* genus. Nucleotide positions 624 to 664 and 683 to 725 of the *M. tuberculosis* gene are hypervariable regions, which are useful for identification purposes (Tortoli 2003). A 441-bp fragment of the *hsp65* gene, known as the Telenti's fragment, is the most used for mycobacteria identification (Telenti, Marchesi et al. 1993).

The *hsp65* gene sequencing shows several advantages over other genes. Since it is present only in a single copy gene, there is no possibility of different nucleotide sequences in the same isolate, as observed with the 16S rRNA gene and the 16S-23S ITS. Another advantage of the *hsp65* gene is the ability to differentiate between closely related species, such as *M. abscessus* and *M. chelonae*, *Mycobacterium szulgai* and *Mycobacterium malmoense; M. kansasii* and *M. gastri* (Joao, Cristovao et al. 2014).

Despite these advantages, some pitfalls of *hsp65* gene have been identified. Since intraspecies similarity is relatively high, from 98 to 100%, a 97% identity cut-off

was recommended. However divergences of 4% were detected in some species such as in *Mycobacterium gordonae*. Therefore a cut-off of 96% associated with results of PCR-Restriction Enzyme Analysis (PRA-*hsp65*) was suggested for accurate species identification (McNabb, Eisler et al. 2004, Joao, Cristovao et al. 2014).

3.4.1.5 Multilocus Sequence Analysis (MLSA)

The disadvantages of DNA sequencing of 16S rRNA, *rpoB*, *hsp65* and ITS sequences in identifying mycobacteria led to analyses of other loci, including *gyrB*, *dnaJ1*, *recA*, *sodA*, *secA1*, *tuf*, *ssrA*, *smpB*, and the 32-kDa protein gene (Soini, Bottger et al. 1994, Zolg and Philippi-Schulz 1994, Blackwood, He et al. 2000, Kasai, Ezaki et al. 2000, Zelazny, Calhoun et al. 2005, Mignard and Flandrois 2007, Yamada-Noda, Ohkusu et al. 2007, Mignard and Flandrois 2008). However, some drawbacks were also identified in these loci. Some of them are not detected in all mycobacterial species and others have low discriminatory power and do not discriminate closely related species (Dai, Chen et al. 2011).

Over the last decades it has been observed that the use of a single gene is not enough to discriminate mycobacterial species. Therefore with the report of the ad hoc committee for the re-evaluation of the species definition in bacteriology a new phylogenetic approach emerged, the Multilocus Sequence Analysis (MLSA) (Stackebrandt, Frederiksen et al. 2002).

MLSA uses several sequences of housekeeping genes. In general, six to eight genes present in all studied taxon as a single copy and not subject to selective pressure are selected for MLSA analysis. The sequences of all selected genes are concatenated and then used to assess clustering patterns, to explore the relationships among strains within a genus and to assign unknown strains to species clusters (Macheras, Roux et al. 2011).

This technique increases the discriminatory power of species identification since the low discriminatory power of individual genes for particular species groups is compensated by others. For example, although *M. chelonae* and *M. abscessus* have the same 16S rRNA gene sequence, *hsp65* is useful to discriminate them. Similarly, *M. senegalense* and *Mycobacterium farcinogenes* have indistinguishable *hsp65*

sequences, but they have distinct 16S rRNA gene sequences (Devulder, Perouse de Montclos et al. 2005).

Several studies have been using the MLSA approach to identify mycobacteria at species level (Devulder, Perouse de Montclos et al. 2005, Mignard and Flandrois 2008, Dai, Chen et al. 2011, Macheras, Roux et al. 2011, Castillo-Rodal, Mazari-Hiriart et al. 2012, Hashemi-Shahraki, Bostanabad et al. 2013). However, there is no well-defined scheme of MLSA and the selected housekeeping genes vary throughout the studies. Moreover, the value of this approach needs to be evaluated in clinical settings as a routine mycobacteria identification method.

3.4.2 PCR (Polymerase Chain Reaction) – Restriction Enzyme Analysis (PRA)

The PRA methodology is based on the amplification of a conserved region, which is subsequently subjected to restriction enzyme analysis. An isolate can be identified comparing its restriction fragment pattern with those generated by known species.

PRA was developed to target genes that are present in all mycobacteria, such as *hsp65* gene (Telenti, Marchesi et al. 1993) and the ITS fragment (Roth, Reischl et al. 2000). For PRA-*hsp65*, the 441-bp Telenti's fragment is amplified and the amplicons are digested using BstEII and HaeIII restriction enzymes. For PRA-ITS, the ITS fragment is amplified and amplicons are digested with HaeIII, CfoI, TaqI, MspI, DdeI, or AvaII restriction enzymes. The use of TaqI is enough for *M. chelonae* and *M. abscessus* identification. The digestion products of both PRA-*hsp65* and PRA-ITS are visualized after electrophoresis in agarose gels and compared to the patterns included in the PRASITE (http://app.chuv.ch/prasite/index.html) for PRA-*hsp65* and published by Roth *et al*. (2000) for PRA-ITS.

Species with overlapping patterns and multiple patterns within a single species have been described and species identification may be ambiguous or erroneous. However, the PRA method is rapid and easy to perform. Moreover the availability of restriction patterns of most clinically relevant mycobacteria species has led to its use as the main molecular method for mycobacteria species identification in reference laboratories of low-income countries, such as Brazil (da Silva Rocha, da Costa Leite

et al. 1999, Leao, Bernardelli et al. 2005, Martin, Uwizeye et al. 2007, Chimara, Ferrazoli et al. 2008).

3.4.3 DNA strip assays

Over the last years, commercial DNA strip assays based on reverse hybridization have been developed for the identification of mycobacteria to the species level, such as the INNO-LiPA® (Innogenetics, Belgium) and GenoType® Mycobacterium assays (Hain, LifeScience, Germany). In these assays a PCR product is hybridized to oligonucleotide probes specific for different mycobacterial species that are immobilized in a nitrocellulose strip. The hybridization pattern of the isolates are then compared with those specified by the manufacturer.

3.4.3.1 INNO-LiPA®

The INNO-LiPA® MYCOBACTERIA (Innogenetics, Ghent, Belgium) was the first commercial DNA probe assay available for identification of the most frequently isolated species of mycobacteria (Tortoli, Mariottini et al. 2003).

This assay was developed in the Institute of Medicine Tropical Prince Leopold, Belgium. Based on the ITS sequence heterogeneity, DNA probes specific for the clinically important mycobacterial species were selected, and a reverse hybridization LiPA (Line Probe Assay) was developed (Portaels, Rigouts et al. 1998). After amplification of the ITS fragment, the biotinylated amplified DNA product is hybridized with specific probes immobilized on membrane strips (Lebrun, Weill et al. 2005).

The second commercial version of this test contains 23 probes that react specifically with M*. tuberculosis* complex (MTB probe), *M. kansasii* (MKA-1, MKA-2, MKA-3), *Mycobacterium xenopi* (MXE), *M. gordonae* (MGO), *Mycobacterium genevense* (MGV), *Mycobacterium simiae* (MSI), *M. marinum-M. ulcerans* (MMU), *Mycobacterium celatum* (MCE), *M. avium* (MAV), *M. intracellulare* (MIN-1, MIN-2), *Mycobacterium scrofulaceum* (MSC), *M malmoense* (MML), *M haemophilum* (MHP), *M. chelonae* (MCH-1, MCH-2, MCH-3), *M. fortuitum* (MFO), and *M. smegmatis* (MSM). One probe is specific for the *Mycobacterium* genus (MYC) and one for the

MAIS complex (reacting with *M avium*, *M. intracellulare*, *M. scrofulaceum* and *M. avium* complex) (Figure 3.1).

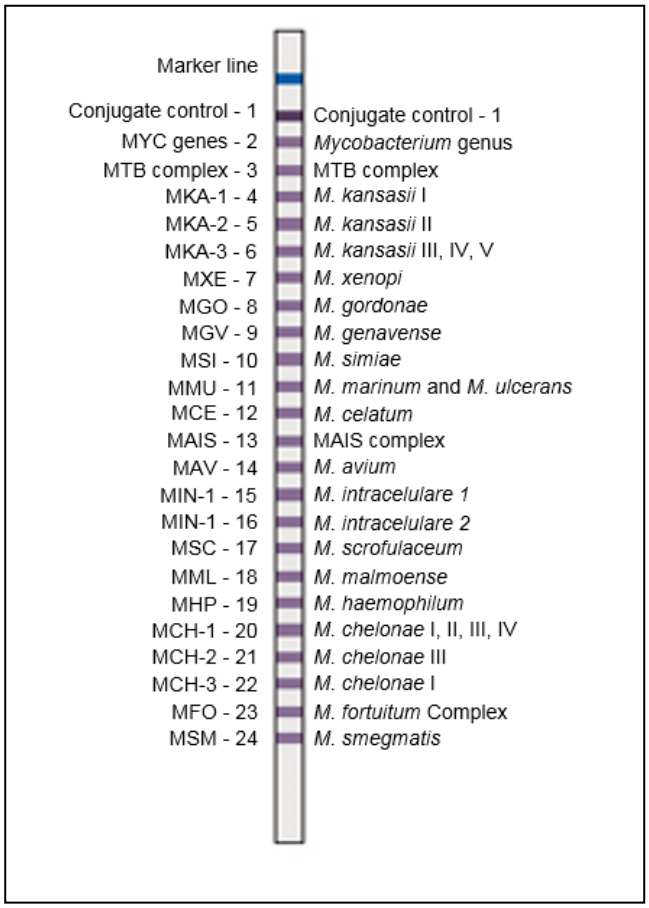

Figure 3.1. Strip design of INNO-LiPA® MYCOBACTERIA (Innogenetics, Ghent, Belgium)

In this assay, isolates of the *M. chelonae-M. abscessus* complex are subdivided into four genotypic clusters (CHI, CHII, CHIII and CHIV) showing the genetic heterogeneity within these taxa. The cluster CHIII is characterized by the hybridization with probes MCH-1 and MCH-2, the cluster CHI with the probes MCH-1 and MCH-3, and the cluster CHII/IV with the probe MCH-1. During the INNO-LiPA® development, distinct features of each genotypic cluster were observed. Cluster CHI isolates showed no clinical importance and were considered to be contaminants from fibroscopes, whereas CHIII isolates were clinically significant and encompassed the specie *M. abscessus* (Portaels, Rigouts et al. 1998, Suffys, da Silva Rocha et al. 2001, Mijs, De Vreese et al. 2002). Strains of clusters CHII and CHIV were isolated from the environment; however they were also considered clinically significant

because some strains caused disease in immunocompromised patients or after dialysis or injection (Portaels, Rigouts et al. 1998).

Tortoli *et al*. evaluated this assay and reported that its sensitivity and specificity was 100% and 94.4%, respectively (Tortoli, Mariottini et al. 2003). However, the probes specific for *M. fortuitum* (MFO), MAIS complex (MAIS) and *M. intracellulare* (MIN-2) showed cross-reaction with several mycobacteria rarely isolated from clinical specimens. Moreover, Lebrun *et al*. reported that several isolates belonging to the MAIS group hybridized only with the complex-specific MAIS probe but not with the specific probes for the complex members (Lebrun, Weill et al. 2005).

3.4.3.2 Genotype® *Mycobacterium* assays

The Genotype® *Mycobacterium* (Hain, LifeScience, Germany) is another available commercial test for mycobacteria identification. There are three different versions of Genotype: the GenoType MTBC for the differentiation of the six species of the *M. tuberculosis* Complex; the GenoType *Mycobacterium* CM (common mycobacteria) for the identification of *M. tuberculosis* Complex plus 13 of the most common NTM species; and the GenoType *Mycobacterium* AS (additional species), for the identification of 16 additional NTM species. Thus, the combined use of CM and AS can distinguish almost 30 different NTM (Figures 3.2 and 3.3) (Richter, Rusch-Gerdes et al. 2006, Russo, Tortoli et al. 2006, Neonakis, Gitti et al. 2008). The target of both AS and CM is the 23S rDNA and therefore only one PCR is needed to perform these assays (Neonakis, Gitti et al. 2008).

Richter *et al*. found 92.6% and 89.9% concordant results of the CM and AS assays respectively, compared to DNA sequencing (Richter, Rusch-Gerdes et al. 2006). According to Russo *et al.*, the sensitivity and specificity of both Genotype® CM and AS are very high (97.9% and 92.4% for CM and 99.3% and 99.4% for AS, respectively) (Russo, Tortoli et al. 2006).

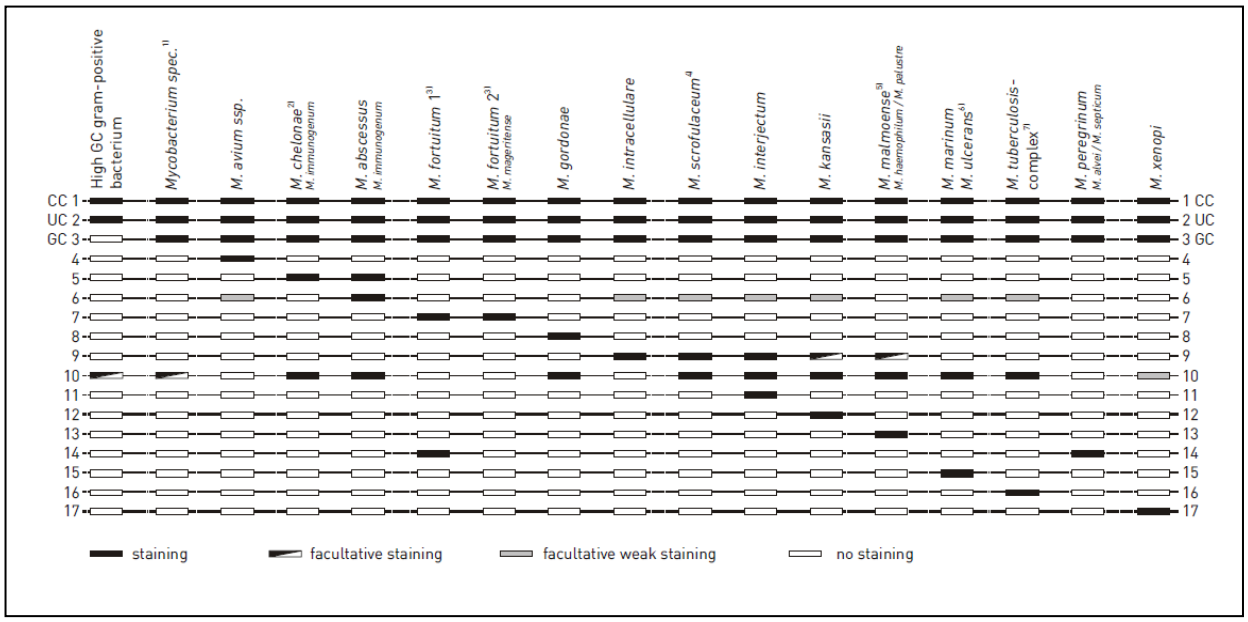

Figure 3.2. Strip design of GenoType® *Mycobacterium* CM (Hain, Nehren, Germany)

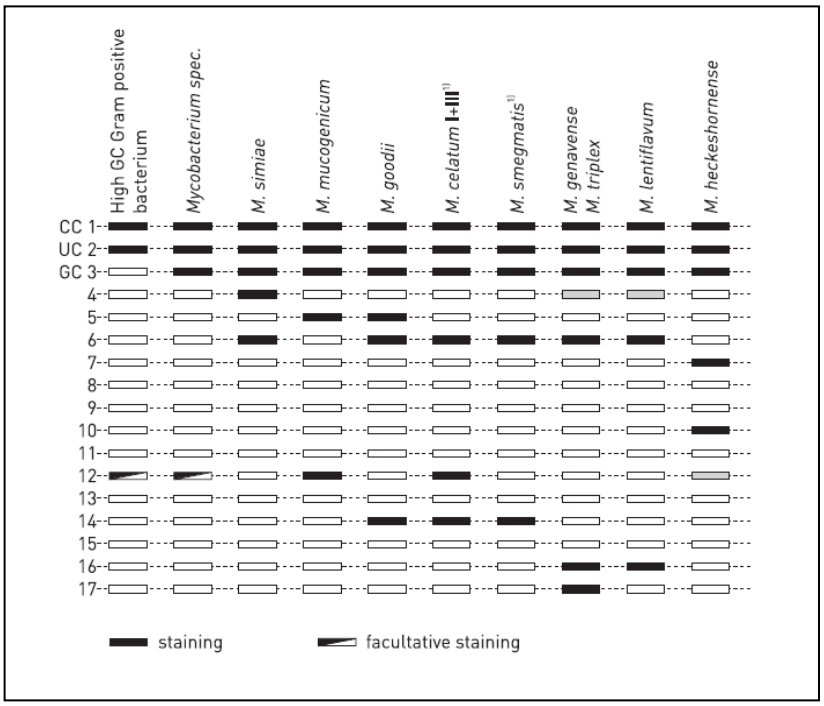

Figure 3.3. Strip design of GenoType® *Mycobacterium* AS (Hain, Nehren, Germany)

The manufacturer declared seven and two ambiguous patterns on the CM and AS strip, respectively. However, the following species were also reported as undistinguishable by GenoType®: *Mycobacterium parascrofulaceum* from *M. scrofulaceum*; *Mycobacterium chimaera* and several intermediate MAC spp. from *M.*

intracellulare; *M. farcinogenes*, *Mycobacterium porcinum*, and *M. senegalense* from *M. fortuitum*. These overlapping patterns is indeed a disadvantage, however most of them are rarely isolated from clinical samples (Russo, Tortoli et al. 2006).

Therefore, GenoType® is a reliable and suitable assay to identify the most frequently encountered species of mycobacteria. Moreover, it is rapid, easy-to-perform and easy-to-interpret. Its implementation in clinical laboratories reduces the number of strains to be identified by mycobacteria reference centers (Richter, Rusch-Gerdes et al. 2006, Russo, Tortoli et al. 2006, Neonakis, Gitti et al. 2008).

Nowadays GenoType® is widely used in clinical laboratories for detection and identification of NTM in European countries. Because of its limitations, if necessary the results can be complemented by 16S rRNA and/or *hsp65* gene sequencing (van der Werf, Kodmon et al. 2014). However, because of their high cost, DNA probe assays and DNA sequencing are not used in clinical laboratories of low-resource countries, such as Brazil. NTM identification in Brazil is still based on some phenotypic tests and PRA-*hsp65* (Chimara, Ferrazoli et al. 2008, Wildner, Bazzo et al. 2014).

4 Drug Susceptibility Testing (DST) for Mycobacteria

Mycobacteria show variable drug susceptibility profiles between different species and also within species. The performance of DST in all NTM isolates recovered from clinical samples is essential for good patient management (Griffith, Aksamit et al. 2007).

The Clinical and Laboratory Standards Institute (CLSI) has standardized procedures for DST performance, including breakpoints for the antimicrobials used for treatment of NTM infections. The antimicrobials tested differ between RGM and SGM. Table 4.1 shows the antimicrobials that should be evaluated for RGM and their respective breakpoints.

CLSI currently recommends Mueller-Hinton broth microdilution method as the gold standard for determining the Minimum Inhibitory Concentration (MIC) of NTM (CLSI 2011). Several laboratories performed it as in-house assays. However, Sensititre™ (Trek Diagnostics, CA, USA), a commercial assay, is also available (van Ingen and Kuijper 2014).

The broth microdilution method is generally performed in a 96-well plate and small volumes of broth with a standardized inoculums are inoculated in each well. The bacteria growth is measured and compared with growth in drug-free control wells. This allows the determination of the minimal concentration of the drug that inhibits the mycobacterial growth (van Ingen and Kuijper 2014).

Table 4.1. Broth Microdilution interpretive criteria for RGM (CLSI, 2011)

Agent	MIC (µg/mL) for category		
	Susceptible	**Intermediate**	**Resistant**
Amikacin (AMK)	≤16	32	≥64
Cefoxitin (FOX)	≤16	32-64	≥128
Ciprofloxacin (CIP)	≤1	2	≥4
Clarithromycin (CLA)	≤2	4	≥8
Doxycycline (DXT)	≤1	2-4	≥8
Minocycline (MIN)	≤1	2-4	≥8
Imipenem	≤4	8-16	≥32
Linezolid	≤8	16	≥32
Meropenem	≤4	8-16	≥32
Moxifloxacin (MF)	≤1	2	≥4
Tobramycin (TOB)	≤2	4	≥8

This technique is easy and reliable; however, some difficulties have been reported, especially with RGM (Broda, Jebbari et al. 2013). In general, the RGM growth in liquid medium form large clumps making the MIC determination by visual turbidity really difficult and subjective. Therefore, the addition of a redox indicator, resazurin, to the broth medium has been proposed (Ramis, Cnockaert et al. 2015). Resazurin is a blue non-fluorescent and non-toxic dye used for the evaluation of cell metabolism since viable cells reduce it into resorufin, which is pink and fluorescent. MIC is defined as the lowest concentration of the drug that prevents the bacterial growth represented by a change in color from blue to pink. Ramis *et al*. showed that resazurin improves the end-point readability of the broth microdilution method and decreases the incubation time from 5 to 3 days shortening the time for results (Ramis, Cnockaert et al. 2015).

5 Description of new species

For several years species of mycobacteria were established according to their phenotypical characteristics only (Butler and Guthertz 2001, Tortoli, Bartoloni et al. 2001). Because it usually leads to the use of the best fit criterion, NTM species were frequently missassigned and new species were infrequently described (Tortoli, Bartoloni et al. 2001). However, the development of highly discriminatory methodologies, such as HPLC of mycolic acids and DNA sequencing has greatly increased the description of new mycobacterial species. A not registered mycolic acid profile and/or a new DNA sequence highlights the possibility of the existence of a new species (Tortoli, Bartoloni et al. 2001, Tortoli 2006).

For the description of a new species it is necessary to follow minimal standards used to determine whether it fulfills the requirements to be assigned to a novel taxon (Ramasamy, Mishra et al. 2014).

The names of new species are considered valid only after publication in the “International Journal of Systematic and Evolutionary Microbiology” (IJSEM), novel prokaryotic taxa official journal. Moreover, before publication of the new species name, a viable culture of the type strain must be deposited in at least two culture collections (Tindall, Rossello-Mora et al. 2010).

Furthermore, for a new species be officially accepted it is necessary to prove that it has not already been described, and that it is distinct from officialy accepted species by performing several methodologies. Moreover, these methodologies should provide the general characteristics of the new species enabling its classifications and identifications (Moore, Mihaylova et al. 2010).

5.1 Polyphasic approach

Prokaryote taxonomists agree that a reliable classification is achieved only when several phenotypic and genomic characteristics are examined. This approach is known as “polyphasic approach” and it is crucial for the delineation of new prokaryotic species. However, as many criteria used were selected empirically, the validity of polyphasic approach is being questioned. Moreover the methodologies used are labor intensive, money consuming, are not accessible to all laboratories and

lack reproducibility (Vandamme, Pot et al. 1996, Rossello-Mora and Amann 2001, Ramasamy, Mishra et al. 2014).

5.1.1 Phenotypic methods

In general, phenotypic analyses provide descriptive information that can be useful for the new taxa recognition (Vandamme, Pot et al. 1996). They include a broad range of methodologies that provide the main morphological, physiological and biochemical characteristics of an organism (Prakash, Verma et al. 2007). Chemotaxonomic techniques such as the pattern of cell wall lipids are also included (Vandamme, Pot et al. 1996, Moore, Mihaylova et al. 2010). As a general recommendation, type strains of the most closely related species should be included in all methodologies performed instead of using data from previously studies (Tindall, Rossello-Mora et al. 2010).

Although the methodologies seem to be simple, the performance of phenotypic tests is a really tedious task. They are time consuming, lack reproducibility and require skill and experience to avoid erroneous observations (Rossello-Mora and Amann 2001, Prakash, Verma et al. 2007). Moreover, an organism can present distinct phenotypic traits according to the environmental conditions and different organisms can present overlapping phenotypic characteristics (Prakash, Verma et al. 2007).

5.1.2 Genotypic methods

Nowadays, the taxonomists agree that all taxonomic information about an organism is incorporated in its genome (Stackebrandt, Frederiksen et al. 2002). Moreover, bacteria are accurately classified when using genome comparisons (Rossello-Mora and Amann 2001).Therefore several genotypic methodologies have been widely used in bacterial taxonomy.

5.1.2.1 DNA G+C content

The genomic DNA G+C content, defined as the proportion of cytosines and guanines within the overall number of nucleotides in the genome, is one of the classical genotypic methods used in the bacterial taxonomy. In a well-defined species, the variability in G+C content percent is below 3% while in a well-defined genus is below 10% (Prakash, Verma et al. 2007).

Until a few years ago, the methods of choice for determining the G+C content were those HPLC-based that allowed direct measurement of the base composition (Mesbah and Whitman 1989, Tindall, Rossello-Mora et al. 2010). Nowadays, it is more often calculated through WGS (Meier-Kolthoff, Klenk et al. 2014).

5.1.2.2 DNA–DNA Hybridization (DDH)

Since DNA G+C content allows only superficial comparisons, a much more accurate method was required. Thus, DDH methodologies were developed (Rossello-Mora and Amann 2001). They have been used since the 1960s and are still an important criterion in bacterial species delineation (Goris, Konstantinidis et al. 2007, Ramasamy, Mishra et al. 2014).

A characteristic property of DNA is its ability of hybridization. Denatured strands of DNA can re-associate reforming native duplex structures. Similarly, DNAs from two distinct organisms can re-associate under standardized conditions. The quantification of the relatedness degree between two organisms can be calculated on the basis of nucleotide sequences identity (Rossello-Mora and Amann 2001). For species delineation, a cutoff of 70% was proposed by Wayne *et al*. (1987) (Wayne*, Brenner et al. 1987). However it is not applicable to all prokaryotic organisms (Goris, Konstantinidis et al. 2007, Ramasamy, Mishra et al. 2014)

DDH can be performed using several distinct techniques, however the methods of choice are those that don´t require radioactive substances for DNA labeling (Tindall, Rossello-Mora et al. 2010). Ezaki *et al*. developed a fluorometric hybridization assay using DNA labeled with photobiotine, a nonradioactive material (Ezaki, Hashimoto et al. 1989). With this methodology, the biotinylated DNA is hybridized with single-stranded unlabeled DNA, which is immobilized on microdilution

plates. After hybridization, the biotinylated DNA is quantitatively detected with streptavidin-conjugated beta-D-galactosidase and a fluorogenic substrate, 4-methylumbelliferyl-betaD-galactopyranoside allowing the determination of the genetic relatedness between two different isolates.

Considerable amounts of high quality DNA are required in all DDH classical techniques, making the whole process time-consuming and labour-intensive (Vandamme, Pot et al. 1996, Goris, Konstantinidis et al. 2007). Moreover, since the comparative nature of the DDH technique, no cumulative databases could be built and single strains could not be analyzed (Gevers, Cohan et al. 2005). Therefore, alternative methods have been searched in order to replace the DDH experiments (Goris, Konstantinidis et al. 2007).

5.1.2.3 DNA sequencing

DNA sequencing has subsequently become the best option among prokaryotic taxonomists for species delimitation and identification purposes. Public database were constructed, from which sequences can be retrieved and compared with sequences of unknown isolates. As a consequence, the bacterial phylogenetic relationships have become easier, faster and more accurate than previously (Richter and Rossello-Mora 2009, Moore, Mihaylova et al. 2010).

The first gene widely used was the 16S rRNA since it is ubiquitously present in all prokaryotes, highly conserved, stable and generally not involved in horizontal gene transfer (Ramasamy, Mishra et al. 2014). A cut-off of 97% is often used to classify bacterial isolates as new species, when compared with species officially accepted. Moreover, it was proposed that the 16S rRNA gene sequence identity could even replace DDH (Stackebrandt and Goebel 1994, Stackebrandt, Frederiksen et al. 2002, Stackebrandt and Ebers 2006).

However, as more sequences were analyzed over the years, several limitations of 16S rRNA as a taxonomic marker were identified, such as its high degree of conservation in some genera, especially in those with closely related organisms, and nucleotides sequence variations in a single organism when multiple rRNA operons are present (Rossello-Mora and Amann 2001, Ramasamy, Mishra et al. 2014).

Therefore it was recommended subsequently that the combination of 16S rRNA gene sequencing and DDH should be used for description of new bacterial species. The performance of DDH is crucial when two isolates have 16S rRNA gene sequence identities above 97% (Tindall, Rossello-Mora et al. 2010).

Because the ad hoc committee recommended the evaluation and application of protein-coding gene sequence analysis to 'genomically circumscribe the taxon species and differentiate it from neighbouring species', the MLSA has become a more and more common method in taxonomic studies (Stackebrandt, Frederiksen et al. 2002, Gevers, Cohan et al. 2005). However, the MLSA scheme must be validated based on DDH data and both MLSA and DDH results should corroborated (Tindall, Rossello-Mora et al. 2010).

5.1.2.4 Whole Genome Sequencing (WGS)

With the introduction of Next-Generation Sequencing (NGS) technology, prokaryotic whole genomes comparison for taxonomic purposes is becoming a reality (Moore, Mihaylova et al. 2010).

The first NGS platform that was widely used was the Roche 454, which was based on pyrosequencing. Another NGS platform, originally developed by Solexa, is Illumina technology, which is based on bridge-amplification. Illumina is provided as different instruments including HiSeq and its lower-throughput called MiSeq (Margulies, Egholm et al. 2005, Bentley 2006).

Since WGS is now fairly accessible, it has been suggested that relatedness indices derived from computational comparison of two genome sequences should replace DDH for taxonomic purposes (Konstantinidis and Tiedje 2005) Among available computational genome relatedness indices, Average Nucleotide Identity (ANI), Genome Blast Distance Phylogeny (GBDP), and Tetranucleotide frequency correlation coefficients (TETRA) are the most used.

ANI is a mean of identity values of all shared orthologous protein coding genes between the two genomes that are being compared. Konstantinidis and Tiedje showed that ANI is a robust measure since it has shown a good correlation with DDH (Konstantinidis and Tiedje 2005). Subsequently, Goris *et al*. refined the ANI calculation (Goris, Konstantinidis et al. 2007). The step of DNA fragmentation in DDH

experiments was computationally simulated by cutting the query genome into fragments of 1020 bp length. Currently, ANI is calculated as a mean identity of all BLASTN matches with at least 30% identity between the two compared genomes. This method is known as ANIb. In 2009, Richter and Rossello-Mora developed the MUMmer software to calculated ANI, which was named ANIm (Richter and Rossello-Mora 2009). This software allows the comparison of large DNA sequences without cutting the query genome. As a consequence it renders faster results than ANIb.

In comparative studies, ANI values between 95%–96% were equivalent to the 70% DDH threshold (Goris, Konstantinidis et al. 2007, Richter and Rossello-Mora 2009). Kim *et al*. confirmed this ANI threshold investigating the ANI values among over six thousand prokaryotic genomes (Kim, Oh et al. 2014). They also demonstrated that this ANI threshold corresponds to 98.65% 16S rRNA sequence identity. Both ANIb and ANIm render very similar results especially when ANI value is above 90% (Richter and Rossello-Mora 2009).

Another strategy named GBDP was introduced as an in silico replacement for DDH experiments. It calculates pairwise intergenomic distances based on the results of a genome-wide homology search using BLASTN and produces a phylogenetic tree (Henz, Huson et al. 2005). It provides a quick and reliable alternative to the wet-lab DDH technique and its improved DDH prediction capability produces classifications which correlate better with the traditional DDH values than do any of the ANI implementations (Meier-Kolthoff, Auch et al. 2013).

In addition to ANI and GBDP, an alignment-free method using Tetranucleotide frequency correlation coefficients (TETRA) was developed. Since there are four types of bases in DNA (A, T, C and G) 256 combinations of tetranucleotide sequences are possible. This method determines the frequency of occurrence of each combination in the genome. In a pairwise comparison, the tetranucleotide frequencies of each genome are plotted, a regression line is obtained and the RSquare value is calculated. It is expected that closely related genomes present high correlation values (RSquare close to 1) since they have similar tetranucleotide frequencies. It was demonstrated that the 95–96% ANI threshold corresponds to TETRA correlation coefficient values above 0.99 (Richter and Rossello-Mora 2009).

TETRA analysis has been reported to be more reliable than the G+C content as a measure of sequence relatedness. Takahashi *et al*. showed that TETRA is

useful for bacterial classification and for phylogenetic relationships estimation among closely related species (Takahashi, Kryukov et al. 2009). Furthermore, since TETRA is an alignment-free method, it can be applied on draft genomes (Ngeow, Wong et al. 2015). In addition, due to the speed of computation analysis, TETRA allows comparisons among high number of genomes (Richter and Rossello-Mora 2009).

5.2 Minimal standards for assignment new species to the *Mycobacterium* genus

According to Recommendation 30b of the International Code of Nomenclature of Bacteria, the analyses should conform at least to the minimal standards (if available) established for the taxon studied (Lapage, Sneath et al. 1992).

In 1992, Levy-Frebault and Portaels proposed that the minimal standards for assignment of a strain to the *Mycobacterium* genus included acid-alcohol fastness, a DNA G+C content in the range from 61 to 71 mol%, and mycolic acid detection with characterization of C22 to C26 pyrolysis esters (Levy-Frebault and Portaels 1992). These authors also proposed the minimal standards for describing new SGM species; however, no recommendations were made regarding RGM.

In order to increase the standardization of methodologies performed for new mycobacteria species description, Tortoli reviewed the phenotypic and genotypic tests used for the description of both new SGM and RGM species in the 1990s (Tortoli 2003). The most common cultural tests included acid-alcohol fastness demonstration, growth temperatures, growth rate, pigmentation type, colony morphology, plus, for RGM, NaCl tolerance, and MacConkey growth. Arylsulfatase, nitrate reduction, Tween 80 hydrolysis, urea hydrolysis, 68°C catalase, and semiquantitative catalase were the most used biochemical tests. Moreover, the performance of lipid analysis using HPLC or other available techniques is recommended since it could be used as an important species identification criterion. Moreover, following the detection of the high G+C content, the characterization of the mycobacterial genome in comparison with other microorganisms, should be investigated in the new species, including 16S rRNA gene sequencing. In spite of its limitations (discussed in section 3.3.1.1), the sequencing of 16S rRNA gene is crucial

for mycobacteria taxonomic studies because it shows the nearest phylogenetic neighbors of the studied isolate (Tortoli, Bartoloni et al. 2001).

After the publication of Tortoli (2003) no additional studies were performed to examine more accurately the minimal standards for mycobacteria species description with focus in the era of genomics. Comparing the recent publications of new RGM species, it is clear that the genotypic tests have a greater impact on the assignment of new species nowadays. The DDH has become less and less popular in recent species description. However, the descriptions of all species that were recently validly named included DNA sequencing of three or more targets, including 16S rRNA, *rpoB*, *hsp65*, and ITS. Moreover, the concatenation of these genes has been shown to increase the robustness of the phylogenetic tree (Brown-Elliott, Wallace et al. 2010, Sahraoui, Ballif et al. 2011, Zhang, Zhang et al. 2012, Guerin-Faublee, Flandrois et al. 2013, Shojaei, Daley et al. 2013, Zhang, Chen et al. 2013, Balcazar, Planas et al. 2014, Fusco da Costa, Fedrizzi et al. 2015, Kim, Kim et al. 2015, Shahraki, Cavusoglu et al. 2015). In 2015, the description of two new mycobacteria species included analysis of WGS. The genomes of *Mycobacterium paraense* nov. sp. isolates were compared, showing that they belong to the same species (Fusco da Costa, Fedrizzi et al. 2015). ANI values were calculated to compare the genomes of *Mycobacterium celeriflavum* nov. sp. and species showing highly similar 16S rRNA gene sequences, to demonstrate that it was a species not yet described (Shahraki, Cavusoglu et al. 2015).

PART III: EXPERIMENTAL WORK

6 Samples and Isolates

This study included 36 isolates of *M. chelonae-M. abscessus* complex comprising a set of isolates of INNO-LiPA® CHII cluster and a set of isolates that failed to match the recognized species patterns.

The INNO-LiPA® assay was developed in the 1990´s. Type strains and several mycobacterial isolates from human, animal and environmental sources were used for its development. These isolates were identified to the species level using tests based on phenotypic characteristics. A set of 9 probes specific to the *M. chelonae-M. abscessus* complex were developed that enabled the subdivision of the isolates of this complex into four clusters (CHI, CHII, CHIII and CHIV) according to their hybridization profile. However, the commercial version of this test used only 3 probes, MCH-1, MCH-2 and MCH-3. Using these probes, the isolates of cluster CHI were characterized by the hybridization with the probes MCH-1 and MCH-3, the cluster CHIII with the probes MCH-1 and MCH-2, and the clusters CHII and CHIV only with the probe MCH-1. During its development, it was verified that *M. abscessus* isolates, including the type strain ATCC 19977, belonged to cluster CHIII (Portaels, Rigouts et al. 1998). Therefore, it was expected that isolates included in the other clusters showed phenotypic characteristics consistent with the species *M. chelonae*, such as the incapacity to grow in the presence of 5% Sodium Chloride (NaCl) at 37^{o}C and the ability to use citrate as the sole carbon source (Kusunoki and Ezaki 1992). However, CHII cluster isolates showed high variability in phenotypic characteristics, suggesting the existence of different taxonomic groups among these isolates. Interestingly, some CHII strains showed phenotypic characteristics consistent with the species *M. abscessus*. In order to explore the CHII cluster internal variability, a set of CHII isolates was kindly provided by Prof. Françoise Portaels including six clinical isolates (five from human and one from animal) from the Institute of Tropical Medicine Prince Leopold, Antwerp, Belgium and twenty-one environmental isolates recovered by Prof. Roland Schulze-Röbbecke at the University of Dusseldorf, Dusseldorf, Germany. One representative strain of each INNO-LiPA® cluster of the *M. chelonae-M. abscessus* complex was also provided: 6410 as the representative strain of cluster CHI, 96-892 of CHII, 8223 of CHIII and 96-443 of CHIV.

Five additional isolates (EPM 10906, EPM 10695, IAL 3785, JAN1 and JAN2) that failed to match the recognized species patterns were also included in this study. They share indistinguishable PRA-*hsp65* patterns – BstEII [bp] (235, 210) and HaeIII [bp] (145, 60, 50) – which is highly similar to *M. abscessus* 1 profile – BstEII [bp] (235, 210) and HaeIII [bp] (145, 70, 60, 50) – but lack the 70 bp HaeIII band. This profile is not present in the PRASITE database. The first two isolates (EPM 10906 and EPM 10695) were obtained in 1999 from corneal specimens of two patients with infectious crystalline keratopathy after LASIK (Laser-Assisted in Situ Keratomileusis). Both isolates were provided by Prof. Denise de Freitas (Departamento de Oftalmologia, Escola Paulista de Medicina, Universidade Federal de São Paulo – EPM-UNIFESP, São Paulo, Brazil). The third isolate (IAL 3785) was recovered in 2007 from a cervical lymph node biopsy, in Ribeirão Preto, São Paulo state, Brazil and sent to the mycobacterial reference laboratory at Instituto Adolfo Lutz (IAL), São Paulo, Brazil for identification. Preliminary analyses based on DNA sequencing of *hsp65*, *rpoB*, ITS and 16S rRNA revealed that the Brazilian isolates were most similar to isolates JAN1 and JAN2 obtained from zebrafish. These isolates were classified as belonging to the *M. chelonae-M. abscessus* complex due to their phenotypic and genotypic characteristics (Whipps, Butler et al. 2007), but they were not assigned to any species of this complex. The JAN1 and JAN2 isolates were kindly provided by Dr. Christopher Whipps from University of Utah, USA for this work.

The following type strains of the *M. chelonae-M. abscessus* complex were also included in this study: *M. abscessus* subsp. *abscessus* ATCC 19977^T, *M. abscessus* subsp. *bolletii* CCUG 50184^T, *M. massiliense* CCUG 48898^T (now classified as *M. abscessus* subsp. *bolletii*), *M. chelonae* ATCC 35752^T, *M. immunogenum* ATCC 700505^T and *M. salmoniphilum* ATCC 13758^T. Although not validly published, "*M. franklinii*" DSM 45524^T was also included in this study.

All type strains and isolates included in this study are listed in Table 6.1.

Table 6.1. Isolates and type strains included in this study.

Isolate	Isolation Source	Procedence	INNO-LiPA®
96-1705	Foot biopsy	ITM, Belgium	CHII
96-1712	Tissue from calf	ITM, Belgium	CHII
96-1717	Tissue from hand	ITM, Belgium	CHII
96-1720	Leg abscess	ITM, Belgium	CHII
96-1724	Leg abscess	ITM, Belgium	CHII

96-1728	Lizard liver tissue	ITM, Belgium	CHII
D16R24	Water work	UD, Germany	CHII
D16R27	Tap water	UD, Germany	CHII
D16Q13	Tap water	UD, Germany	CHII
D16Q14	Tap water	UD, Germany	CHII
D16Q16	Water work	UD, Germany	CHII
D16Q19	Water work	UD, Germany	CHII
D16Q20	Water work	UD, Germany	CHII
D16Q24	Tap water	UD, Germany	CHII
D17A2	Water work	UD, Germany	CHII
D16R4	Tap water	UD, Germany	CHII
D16R12	Surface water	UD, Germany	CHII
D16R18	Tap water	UD, Germany	CHII
D16Q15	Tap water	UD, Germany	CHII
D16R2	Tap water	UD, Germany	CHII
D16R3	Tap water	UD, Germany	CHII
D16R7	Tap water	UD, Germany	CHII
D16R9	Tap water	UD, Germany	CHII
D16R10	Surface water	UD, Germany	CHII
D16R14	Tap water	UD, Germany	CHII
D16R19	Surface water	UD, Germany	CHII
D16R20	Surface water	UD, Germany	CHII
6410*	-	ITM, Belgium	CHI
96-892*	-	ITM, Belgium	CHII
8223*	-	ITM, Belgium	CHIII
96-443*	-	ITM, Belgium	CHIV
EPM 10695	LASIK surgery	EPM-UNIFESP, Brazil	ND
EPM 10906	LASIK surgery	EPM-UNIFESP, Brazil	ND
IAL 3785	Linfonode	IAL, Brazil	ND
JAN1	Zebrafish	University of Utah, USA	ND
JAN2	Zebrafish	University of Utah, USA	ND
M. abscessus subsp. *abscessus*	-	ATCC 19977^{T}	CHIII
M. abscessus subsp. *bolletii*	-	CCUG 50184^{T}	ND
M. massiliense[#]	-	CCUG 48898^{T}	ND
M. chelonae	-	ATCC 35752^{T}	CHII/IV
M. immunogenum	-	ATCC 700505^{T}	ND
M. salmoniphilum	-	ATCC 13758^{T}	ND
M. franklinii	-	DSM 45524^{T}	ND

(EPM-UNIFESP): Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil; (UD): University of Dusseldorf, Dusseldorf, Germany; (ITM): Institute of Tropical Medicine Prince Leopold, Antwerp, Belgium; (IAL): Instituto Adolfo Lutz, São Paulo, Brazil; (ND): not determined
*representative strains of each cluster used during the development of INNO-LiPA® assay
[#]now classified as *M. abscessus* subsp. *bolletii*

7 Preliminary Identification of the Isolates

7.1 Introduction

In the first phase of this study, all isolates were preliminarily characterized using the commercial test Genotype® *Mycobacterium* CM and AS and the PCR Restriction Enzyme Analysis of the *hsp65* gene (PRA-*hsp65*) and 16S-23S ITS (PRA-ITS)

7.2 Materials and Methods

7.2.1 DNA extraction

Cultures were grown on Lysogeny Broth (LB) agar incubated at 30^{o}C. DNA was prepared by boiling one loop full of bacteria for 10 min in 300 µL of TET (10 mM Tris, 1 mM EDTA, 1% Triton X-100 [pH 8.0]) followed by centrifugation at 14,000 g for 2 min.

7.2.2 GenoType® *Mycobacterium* CM and AS

GenoType® CM and AS were performed according to the manufacturer's instructions (Hain, Nehren, Germany).

7.2.3 PRA-*hsp65* and PRA-ITS

For PRA-*hsp65*, a 441-bp fragment of the *hsp65* gene was amplified as described by Telenti *et al*. with some modifications (Telenti, Marchesi et al. 1993). The composition of the PCR mixture (50 µL) was 140 µM (each) dNTP, 0.4 µM Tb11 (5' ACCAACGATGGTGTGTCCAT), 0.4 µM Tb12 (5' CTTGTCGAACCGCATACCCT), 1.5 mM $MgCl_2$, 10% reaction buffer (150 mM Tris-HCl, pH 8.75, 500 mM KCl and 1% Triton X-100), 5% glycerol and 0.6 U Taq polymerase. Five microliters of DNA lysate were added to each reaction tube. The reaction was subjected to an initial denaturation at 96^{o}C for 5 minutes, followed by 45

cycles of amplification (1 min at 95°C, 1 min at 65°C, 1 min at 72°C), and 10 min of final extension at 72°C. Subsequently, amplicons were digested in two separate tubes with BstEII and HaeIII restriction enzymes at 60^{o}C and 37^{o}C, respectively.

For PRA-ITS, the ITS fragment was amplified as described by Roth *et al*. with some modifications (Roth, Reischl et al. 2000). The composition of the PCR mixture (30 µL) was 200 µM (each) dNTP, 0.4 µM Sp1 (5' ACCTCCTTTCTAAGGAGCACC), 0.4 µM Sp2 (5' GATGCTCGCAACCACTATCCA), 1.5 mM $MgCl_2$, 10% reaction buffer (150 mM Tris-HCl, pH 8.75, 500 mM KCl and 1% Triton X-100) and 0.5 U Taq polymerase. Ten microliters of DNA lysate were added to each reaction tube. The reaction was subjected to an initial denaturation at 96^{o}C for 5 minutes, followed by 45 cycles of amplification (1 min at 94°C, 1 min at 60°C, 1 min at 72°C), and 10 min of final extension at 72°C. Amplicons were digested with TaqI restriction enzyme at 65^{o}C.

Both PRA-*hsp65* and PRA-ITS digestion products were visualized in 3% agarose gels (MetaPhor™ Agarose, Lonza, USA) stained with ethidium bromide after electrophoresis, using 50-bp and 25-bp ladder as molecular size standards. The restriction fragment sizes were estimated using the BioNumerics program version 7.6 (Applied Maths, Sint-Martens-Latem, Belgium) and compared to the patterns included in the PRASITE (http://app.chuv.ch/prasite/index.html) for PRA-*hsp65* and published by Roth, Reischl *et al*. (2000) for PRA-ITS.

7.3 Results and discussion

Using Genotype® CM all isolates showed hybridization with probes 5 and 10, with exception of isolates 8223 and 6410 that showed hybridization with probes 5, 6 and 10. According to the manufacturer, the first hybridization profile corresponds to *M. chelonae* and the second to *M. abscessus*.

The type strains *M. abscessus* subsp. *abscessus* ATCC 19977^{T}, *M. abscessus* subsp. *bolletii* CCUG 50184^{T} and *M. massiliense* CCUG 48898^{T} showed hybridization with probes 5, 6 and 10 (hybridization profile of *M. abscessus*/*M. immunogenum*), while *M. chelonae* ATCC 35752^{T}, *M. immunogenum* ATCC 700505^{T}, *M. salmoniphilum* ATCC 13756^{T} and "*M. franklinii*" DSM 45524^{T} showed

hybridization with probes 5 and 10 (hybridization profile of *M. chelonae*/*M. immunogenum*).

Using Genotype *Mycobacterium* AS, all isolates and type strains showed the same hybridization profile (hybridization with probe 12) that corresponds to a species different from the mycobacteria species identifiable by this assay.

The performance of GenoType® assay confirmed that all isolates included in this study belong to the *M. chelonae*-*M. abscessus* complex.

According the PRA results, the isolates were separated into groups wherein the isolates of each group showed the same restriction profile of both PRA-*hsp65* and PRA-ITS (Table 7.1 and Figure 7.1).

Table 7.1. Restriction profiles of PRA-*hsp65* and PRA-ITS of all isolates included in this study and their identification based on PRASITE and Roth et al., 2000, respectively. The horizontal lines separate the groups that have the same restriction profile of PRA-*hps65* and PRA-ITS. The two last groups contain four isolates used to develop INNO-LiPA® and the *M. chelonae*- *M. abscessus* complex type strains, respectively.

Isolate	PRA-*hsp65*			PRA-ITS	
	BSTEII	HAEIII	Identification	TaqI	Identification
96-1712	320/130	200/60/55/50	*M. chelonae* type 1	No restriction	*M. chelonae* type I
D16R4	320/130	200/60/55/50	*M. chelonae* type 1	No restriction	*M. chelonae* type I
96-1717	320/130	200/60/55/50	*M. chelonae* type 1	140/120	*M. chelonae* type II
96-1720	320/130	200/60/55/50	*M. chelonae* type 1	140/120	*M. chelonae* type II
96-1724	320/130	200/60/55/50	*M. chelonae* type 1	140/120	*M. chelonae* type II
96-1728	320/130	200/60/55/50	*M. chelonae* type 1	140/120	*M. chelonae* type II
D16Q24	320/130	200/60/55/50	*M. chelonae* type 1	140/120	*M. chelonae* type II
D16R2	320/130	200/60/55/50	*M. chelonae* type 1	140/120	*M. chelonae* type II
D16R3	320/130	200/60/55/50	*M. chelonae* type 1	140/120	*M. chelonae* type II
D16R7	320/130	200/60/55/50	*M. chelonae* type 1	140/120	*M. chelonae* type II
D16R9	320/130	200/60/55/50	*M. chelonae* type 1	140/120	*M. chelonae* type II
D16R10	320/130	200/60/55/50	*M. chelonae* type 1	140/120	*M. chelonae* type II
D16R14	320/130	200/60/55/50	*M. chelonae* type 1	140/120	*M. chelonae* type II
D16R19	320/130	200/60/55/50	*M. chelonae* type 1	140/120	*M. chelonae* type II
D16R20	320/130	200/60/55/50	*M. chelonae* type 1	140/120	*M. chelonae* type II
96-1705	320/130	200/55/50	*M. chelonae* type 2*	No restriction	*M. chelonae* type I
D16Q13	320/130	200/55/50	*M. chelonae* type 2*	225/30	*M. abscessus*
D16Q14	320/130	200/55/50	*M. chelonae* type 2*	225/30	*M. abscessus*
D16Q16	320/130	200/55/50	*M. chelonae* type 2*	225/30	*M. abscessus*
D16Q20	320/130	200/55/50	*M. chelonae* type 2*	225/30	*M. abscessus*
D16R24	320/130	200/55/50	*M. chelonae* type 2*	225/30	*M. abscessus*
D17A2	320/130	200/55/50	*M. chelonae* type 2*	225/30	*M. abscessus*

D16Q15	320/130	200/115/60/50	Not registered	120/105/30	*M. chelonae* type III
D16R12	320/130	200/115/60/50	Not registered	120/105/30	*M. chelonae* type III
D16R18	320/130	200/115/60/50	Not registered	120/105/30	*M. chelonae* type III
D16Q19	320/130	200/70/60/55	*M. immunogenum* type 2	225/30	*M. abscessus*
D16R27	320/130	200/70/60/55	*M. immunogenum* type 2	225/30	*M. abscessus*
EPM 10906	235/210	145/60/55/50	Not registered	225/30	*M. abscessus*
EPM 10695	235/210	145/60/55/50	Not registered	225/30	*M. abscessus*
IAL 3785	235/210	145/60/55/50	Not registered	225/30	*M. abscessus*
JAN1	235/210	145/60/55/50	Not registered	225/30	*M. abscessus*
JAN2	235/210	145/60/55/50	Not registered	225/30	*M. abscessus*
6410 (CHI)	310/130	200/70/60/55	*M. immunogenum* type 2	No restriction	*M. chelonae* type I
96-892 (CHII)	320/130	200/55/50	*M. chelonae* type 2*	225/30	*M. abscessus*
8223 (CHIII)	235/210	145/70/60/55	*M. abscessus* type 1	225/30	*M. abscessus*
96-443 (CHIV)	320/130	200/60/55/50	*M. chelonae* type 1	140/120	*M. chelonae* type II
ATCC 19977^T	235/210	145/70/60/50	*M. abscessus* type 1	225/30	*M. abscessus*
CCUG 50184^T	235/210	200/70/60/50	*M. abscessus* type 2	225/30	*M. abscessus*
CCUG 48898^T	235/210	200/70/60/50	*M. abscessus* type 2	225/30	*M. abscessus*
ATCC 35752^T	320/130	200/60/55/50	*M. chelonae* type 1	No restriction	*M. chelonae* type I
ATCC 700505^T	320/130	145/70/60/55	*M. immunogenum* type 1	150/125	Not registered
ATCC 13758^T	320/130	200/60/55/50	*M. chelonae* type 1	120/105/30	*M. chelonae* type III
DSM 45524^T	320/130	200/70/60/55	*M. immunogenum* type 2	225/30	*M. abscessus*

*not present in the PRASITE database

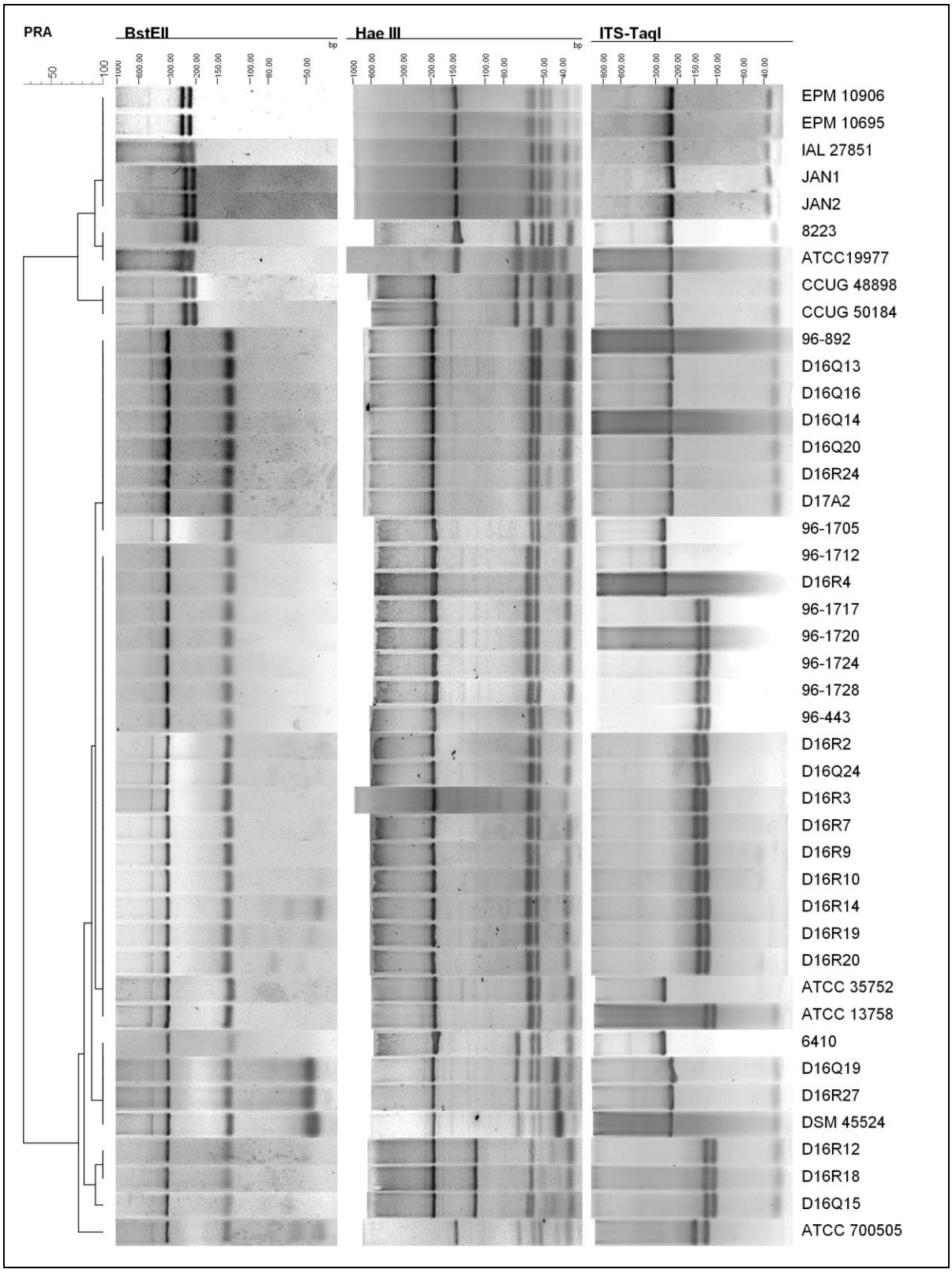

Figure 7.1. Gel electrophoresis of PCR Restriction Enzyme Analysis of the *hsp65* gene (PRA-*hsp65*) and the 16S-23S Internal Transcribed Spacer (PRA-ITS) of all isolates and type strains included in this study.

The representative strains of the CHI, CHII, CHIII and CHIV cluster used during the development of INNO-LiPA® showed different restrictions profiles, confirming that they represent different taxa. The same was observed with the *M. chelonae-M. abscessus* type strains, except for *M. abscessus* subsp. *bolletii* and *M. massiliense*, which share the same PRA patterns. By the current accepted classification both are grouped in the same subspecies, that of *M. abscessus* subsp. *bolletii.*

Fifteen isolates (96-1712, D16R4, 96-1717, 96-1720, 96-1724, 96-1728, D16Q24, D16R2, D16R3, D16R7, D16R9, D16R10, D16R14, D16R19, D16R20) and the representative strain of CHIV cluster (96-443) showed the *M. chelonae* type 1 PRA-*hsp65* profile and two different restriction profiles of PRA-ITS using the enzyme TaqI (*M. chelonae* type I and type II).

One isolate (96-1705) showed a novel PRA-*hsp65* profile similar to that of *M. chelonae* type 1 but lacking the 60-bp Hae III restriction band, named here *M. chelonae* type 2 and the PRA-ITS profile of *M. chelonae* type I.

Seven isolates (96-892, D16Q13, D16Q14, D16Q16, D16Q20, D16R24 and D17A2) were identified as by PRA-*hsp65* as *M. chelonae* type 2 and as *M. abscessus* by PRA-ITS. This PRA patterns were shared by the representative strain of CHII cluster (96-892).

Isolates D16Q15, D16R12 and D16R18 were identified as *M. chelonae* type III by PRA-ITS and showed a new PRA-*hsp65* restriction profile, not registered in the PRASITE database.

D16Q19 and D16R27 were identified as *M. immunogenum* type 2 by PRA-*hsp65* and *M. abscessus* by PRA-ITS. This profile was shared by "*M. franklinii*" DSM 45524.

Five isolates (EPM 10906, EPM 10695, IAL 3785, JAN1 and JAN2) were identified as *M. abscessus* by PRA-ITS and showed a new PRA-*hsp65* profile, not registered in the PRASITE database.

During INNO-LiPA® development, variability in phenotypic tests of CHII cluster isolates was observed, suggesting the existence of different taxonomic groups among these isolates. Here we demonstrated that the CHII cluster isolates were distributed in different groups according to their PRA-*hsp65* and PRA-ITS restriction profiles demonstrating high genotypic variability. The groups formed by PRA-*hsp65*

and PRA-ITS profiles can represent distinct taxa. However a conclusive classification was not achieved for all isolates.

Since the taxonomic position of several isolates remained doubtful, a polyphasic analysis was subsequently carried out in order to explore the internal variability of CHII cluster isolates and to clarify the taxonomic position of the isolates with no conclusive species identification. First of all, the isolates were characterized using phenotypic analyses traditionally used for the identification of mycobacteria, based on culture characteristics and biochemical features; drug susceptibility testing; and DNA sequencing of the 16S rRNA, *hsp65* and *rpoB* genes and the 16S-23S ITS. Then some isolates were submitted to DNA-DNA hybridization experiments leading to the description of *M. franklinii* sp. nov. (CHAPTER 8) and *M. saopaulense* sp. nov. (CHAPTER 9), respectively. Even after the performance of several phenotypic and molecular methodologies, the taxonomic classification of several isolates was not achieved. Therefore, the CHII cluster isolates were submitted to WGS and pairwise genome comparisons in order to clarify their classification and taxonomic position and to explore their internal variability (CHAPTER 10).

8 *Mycobacterium franklinii* sp. nov., a species closely related to members of the *Mycobacterium chelonae-Mycobacterium abscessus* group

Redraft from: **Nogueira CL**, Simmon KE, Chimara E, Cnockaert M, Palomino JC, Martin A, Vandamme P, Brown-Elliott BA, Wallace R Jr, Cardoso Leão S. *Mycobacterium franklinii* sp. nov., a species closely related to members of the *Mycobacterium chelonae-Mycobacterium abscessus* group. International Journal of Systematics and Evolutionary Microbiology 65 (7): 2148-53, 2015.

Abstract

Two isolates from water, D16Q19 and D16R27, were shown to be highly similar in their 16S rRNA, 16S-23S internal transcribed sequence (ITS), *hsp65* and *rpoB* gene sequences to *Mycobacterium franklinii* DSM 45524^T, described but not validly named in 2011. They are all nonpigmented rapid growers and are phenotypically and genetically related to the *Mycobacterium chelonae-Mycobacterium abscessus* group. Extensive characterization by phenotypic analysis, biochemical tests, drug susceptibility testing, PCR-Restriction Enzyme Analysis of the *hsp65* gene and ITS, DNA sequencing of housekeeping genes and DNA-DNA hybridization demonstrated that *M. franklinii* DSM 45524^T, D16Q19 and D16R27 belong to a single species that is separated from other members of the *M. chelonae-M. abscessus* group. On the basis of these results we propose the formal recognition of *Mycobacterium franklinii* sp. nov. Strain DSM 45524^T (=ATCC BAA-2149^T) is the type strain.

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA, *hsp65*, *rpoB* and ITS sequences of isolate D16R27 are KM392061, KM392060, KM392057 and KM392062, respectively. Those for the partial *hsp65* and *rpoB* gene sequences of *M. franklinii* DSM 45524^T are KM392059 and KM392056, respectively and for the partial *rpoB* gene sequence of *M. salmoniphilum* ATCC 13758^T is KM392058.

The *Mycobacterium chelonae-Mycobacterium abscessus* group comprises closely related rapidly growing mycobacteria. They are widespread saprophytes that can cause several opportunistic infections in humans, ranging from localized abscesses to pulmonary and disseminated disease (Wallace Jr, Swenson et al. 1983, Brown-Elliott and Wallace 2002). Skin and soft tissue infections caused by members of *M. chelonae-M. abscessus* group have been increasingly reported in recent years, representing a serious public health problem in some settings. The ubiquitous distribution of these organisms facilitates the contamination of medical equipment and solutions, which may be associated with the growing number of therapeutic interventions leading to nosocomial infections and outbreaks (Tortoli 2009, **Brown-Elliott** and **Wallace Jr.** 2012a).

Until 2007, the *M. chelonae-M. abscessus* group was composed of *Mycobacterium chelonae*, *Mycobacterium abscessus* (Kusunoki and Ezaki 1992), *Mycobacterium immunogenum* (Wilson, Steingrube et al. 2001), *Mycobacterium massiliense* (Adekambi, Reynaud-Gaubert et al. 2004a, Adékambi, Reynaud-Gaubert et al. 2006), *Mycobacterium bollet*ii (Adekambi, Berger et al. 2006) and *Mycobacterium salmoniphilum* (Whipps, Butler et al. 2007). Recently, this group has undergone taxonomic changes, including the unification of *M. abscessus*, *M. massiliense* and *M. bolletii* in a single species (*M. abscessus*) with two subspecies (*M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii*). Strains formerly named *M. massiliense* were reclassified as *M. abscessus* subsp. *bolletii (Leao, Tortoli et al. 2009, Leao, Tortoli et al. 2011)*. Although beyond the scope of this paper, recently whole genomic sequencing has provided evidence that there are indeed three taxonomic groups within *M. abscessus*. It is not clear yet how this will affect the 2011 reclassification (Tettelin, Davidson et al. 2014).

In 2011, Simmon *et al.* studied a group of clinical isolates from Pennsylvania, and primarily the Northeastern United States, that were initially misidentified as *M. chelonae* with an atypical antimicrobial drug susceptibility profile (Simmon, Brown-Elliott et al. 2011). This study included the proposal of *Mycobacterium franklinii* as a new member of *M. chelonae-M. abscessus* group, but this name has not been validly published.

During taxonomic studies of isolates from the collection of Prof. Françoise Portaels (Antwerp, Belgium), two isolates (D16Q19 and D16R27) showed high

similarity with *M. franklinii* by sequencing of 16S rRNA, *hsp65*, *rpoB* and the 16S–23S internal transcribed spacer (ITS). Strains D16Q19 and D16R27 were isolated in Germany by Prof. Roland Schulze-Röbbecke (Düsselforf, Germany) from water work and tap water, respectively.

The aim of the present study was to examine the taxonomic position of these two isolates and *M. franklinii* DSM 45524^T using phenotypic and genotypic tests. The results were compared with those displayed by other members of *M. chelonae-M. abscessus* group, *M. abscessus* subsp. *abscessus* ATCC 19977^T, *M. abscessus* subsp. *bolletii* CCUG 50184^T, *M. chelonae* ATCC 35752^T, *M. immunogenum* ATCC 700505^T and *M. salmoniphilum* ATCC 13758^T.

Cultures were grown on solid-phase media including Middlebrook 7H10 and Luria-Bertani agar and in liquid medium including Muller-Hinton and Luria-Bertani broth with 1% Tween 80. All cultures were incubated at 28–30^{o}C. Microscopic examination of isolated colonies by Zhiel-Neelsen staining showed that isolates D16Q19, D16R27 and *M. franklinii* DSM 45524^T were acid-fast bacilli. Pigment production, single-source carbon utilization (mannitol, inositol and citrate), growth at 26^{o}C and 37^{o}C and the ability to grow in the presence of 5% NaCl, 0.2% picric acid, para-nitrobenzoic acid (PNB) and nitrite were performed on 7H10 medium. Nitrate reduction and Tween 80 hydrolysis were also examined. The results of these tests were read after 7 and 14 days incubation at 28^{o}C, except for Tween 80 hydrolysis, which was read after 5 and 10 days, as described in standard protocols for biochemical testing of mycobacteria (Tsukamura 1984, Kent and Kubica 1985).

The isolates D16Q19, D16R27 and *M. franklinii* DSM 45524^T exhibited indistinguishable phenotypic and biochemical characteristics, which are indicated in Table S8.1. Colonies that appeared on Middlebrook 7H10 and Luria-Bertani agar after aerobic incubation were nonpigmented. On these solid media, visible growth required 3 to 5 days at 25^{o}C to 37^{o}C; optimum growth occurred at 28^{o}C. The bacterial cells showed tendency to form large clumps when grown in liquid media and the use of 0.1% Tween 80 helped to obtain dispersed growth. They grew in the presence of 5% NaCl, picric acid, PNB and nitrite. They were negative for nitrate reduction and Tween hydrolysis and growth did not occur when mannitol or inositol was used as single-source of carbon. D16Q19, D16R27, *M. chelonae* ATCC 35752^T and *M. immunogenum* ATCC 700505^T grew in the presence of

citrate but not *M. franklinii* DSM 45524^T or the other type strains of the *M. chelonae-M. abscessus* group. These phenotypic and biochemical tests were not useful for distinguishing D16Q19, D16R27 and *M. franklinii* DSM 45524^T from other members of the *M. chelonae-M. abscessus* group.

Antimicrobial susceptibility testing was performed in cation-supplemented Mueller–Hinton broth by the microdilution method, according to the recommendations of the Clinical and Laboratory Standards Institute for rapidly growing mycobacteria (CLSI 2011). The antimicrobials tested were amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxycycline, minocycline, moxifloxacin and tobramycin. According to CLSI interpretative standards, isolates D16Q19, D16R27 and *M. franklinii* DSM 45524^T were susceptible to amikacin, ciprofloxacin, doxycycline, minocycline, and moxifloxacin. D16Q19 and D16R27 were susceptible to clarithromycin at 3 days incubation but extended clarithromycin MICs (14 days incubation) was not performed. Susceptibility of D16Q19 and D16R27 to cefoxitin was intermediate and they were resistant or intermediate to tobramycin while *M. franklinii* DSM 45524^T was susceptible to both drugs (Table 8.1). The results obtained with cefoxitin were in agreement with results obtained by Simmon & Brown-Elliott *et al.*, who reported intermediate and susceptible rates of 88% and 22%, respectively, for *M. franklinii* isolates (Simmon, Brown-Elliott et al. 2011). D16Q19, D16R27 and *M. franklinii* DSM 45524^T were more susceptible to antimicrobial drugs than the other members of the *M. chelonae-M. abscessus* group, but studies of larger strain numbers have shown greater MIC variability than exhibited by these reference type strains (Table 8.1).

GenoType® *Mycobacterium* common mycobacteria (CM) and additional species (AS) assays (Hain Lifescience, Nehren Germany), two commercial DNA strip assays for mycobacteria identification, were performed according to manufacturer's instructions. Using GenoType® CM, the isolates D16Q19, D16R27 and *M. franklinii* DSM 45524^T showed the *M. chelonae* profile (hybridization with probes 5 and 10). They showed the same GenoType® AS profile, hybridization with probe 12, which assigns the isolates to a species of mycobacteria different from the eighteen species identifiable with the AS strip.

Table 8.1. Antimicrobial susceptibility results with isolates and type strains included here and 25 CV* isolates from the previous study (Simmon, Brown-Elliott et al. 2011)

Isolates/strains: 1, D16Q19; 2, D16R27; 3, *M. franklinii* DSM 45524^T; 4, Range of results of 25 CV* isolates; 5, *M. abscessus* subsp. *abscessus* ATCC 19977^T; 6, *M. abscessus* subsp. *bolletii* CCUG 50184^T; 7, *M. chelonae* ATCC 35752^T; 8, *M. immunogenum* ATCC 700505^T; 9, *M. salmoniphilum* ATCC 13758^T. The antimicrobial susceptibility breakpoints were those established by CLSI (2011) for rapidly growing mycobacteria.

Drugs	MIC (µg ml^{-1})								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Amikacin	8	16	≤ 4	8-32 (n=25)	8	16	8	≤ 4	16
Ciprofloxacin	≤ 0.25	≤ 0.25	0.5	≤0.25-8 (n=25)	4	8	0.5	1	1
Clarithromycin (3 days)	≤ 0.5	≤ 0.5	≤ 0.5	≤0.12-1 (n=25)	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5
Clarithromycin (14 days)	ND	ND	ND	<2 (n=8)	ND	ND	ND	ND	ND
Doxycycline	≤ 0.25	≤ 0.5	≤ 0.25	≤0.25->32 (n=12)	>32	>32	4	>32	>32
Cefoxitin	64	64	16	16-64 (n=25)	64	32	512	256	512
Tobramycin	4	16	2	4-16 (n=24)	8	16	2	4	4
Minocycline	1	≤ 0.25	≤ 0.25	≤0.5-16 (n=22)	8	16	2	8	2
Moxifloxacin	0.5	1	1	0.5-8 (n=11)	8	8	≤ 0.25	1	2

(ND) not determined, (n) number of isolates tested by Simmon & Brown-Elliott *et al*. (Simmon, Brown-Elliott et al. 2011)
*Simmon, Brown-Elliott et al. (2011) labeled *M. franklinii* isolates as CV indicating *M. chelonae* variant

For molecular identification by PCR Restriction Enzyme Analysis (PRA) of the *hsp65* gene and ITS, the DNA was prepared by boiling one loop full of bacteria for 10 min in 300 µL of TET (10 mM Tris, 1 mM EDTA, 1% Triton X-100 [pH 8.0]) followed by centrifugation at 14,000 *g* for 2 min. For PRA-*hsp65*, a 441-bp fragment of the *hsp65* gene was amplified using primers Tb11 and Tb12 (Table S2), as previously described (Telenti, Marchesi et al. 1993). The amplicons were digested in two separate tubes with BstEII and HaeIII restriction enzymes. For PRA-ITS, the ITS fragment was amplified using primers Sp1 and Sp2 (Table S8.2), as previously described (Roth, Reischl et al. 2000). ITS amplicons were digested with TaqI restriction enzyme. The digestion products of both PRA-*hsp65* and PRA-ITS were visualized after electrophoresis in 3% agarose gels stained with ethidium bromide, using 50-bp ladder as the molecular size standard. The restriction fragment sizes were estimated using BioNumerics program version 5.1 (Applied Maths, Sint-Martens-Latem, Belgium) and compared to the patterns included in the PRASITE (http://app.chuv.ch/prasite/index.html) for PRA-*hsp65*

and published by Roth *et al.* (2000) for PRA-ITS. Isolates D16Q19, D16R27 and *M. franklinii* DSM 45524 showed indistinguishable PRA-*hsp65* patterns – BstEII [bp] (320, 130) and HaeIII [bp] (200, 70, 60, 55). They showed the same PRA-ITS pattern – TaqI [bp] (225, 30), which is common to *M. abscessus*. Both assays, Genotype® and PRA were not useful for distinguishing D16Q19, D16R27 and *M. franklinii* DSM 45524^T from other members of the *M. chelonae-M. abscessus* group (data not shown).

Partial sequences of 16S rRNA (Harmsen, Dostal et al. 2003, Adekambi and Drancourt 2004b, Gomila, Ramirez et al. 2007), *hsp65* (Telenti, Marchesi et al. 1993), *rpoB* (Adekambi, Colson et al. 2003) and ITS (Roth, Reischl et al. 2000) were obtained with isolates D16Q19 and D16R27. The primers used for PCR amplification and sequencing are listed in Table S8.2. PCR products were purified using QIAquick PCR purification Kit (Qiagen, Germany). Dideoxy sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and run in ABI PRISM 3100 DNA Analyzer (Applied Biosystems). Sequences from both isolates D16Q19 and D16R27 shared 100% identity and only the sequences from isolate D16R27 were deposited in the GenBank database. The corresponding sequences of the *M. chelonae-M. abscessus* group type strains were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). The *hsp65* and *rpoB* genes of *M. franklinii* DSM 45524^T and the *rpoB* gene of *M. salmoniphilum* ATCC 13758^T were resequenced, as the sequences deposited in the GenBank database were incomplete. All sequences of isolates D16R27 and D16Q19 showed the highest sequence similarity with the corresponding sequences of *M. franklinii* DSM 45524^T, suggesting that they belong to the same taxon (Table S8.3). Sequence alignments and phylogenetic trees were constructed by using neighbor-joining method with Kimura 2-parameter distance correction model and 1,000 bootstrap replications in MEGA6 (Tamura, Stecher et al. 2013) (Figure 8.1).

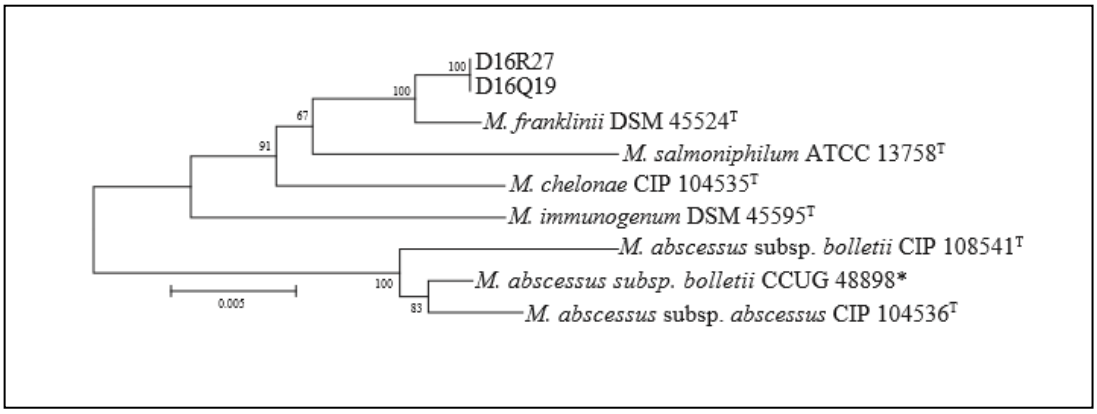

Figure 8.1. Neighbor-joining tree of concatenated 16S rRNA gene, ITS, *hsp65* gene and *rpoB* gene from *M. chelonae-abscessus* group type strains and D16Q19 and D16R27 isolates. The 16S rRNA culture collection strain designation is shown next to the species name. Genbank accession of the 16S rRNA, ITS, *hsp65* and *rpoB* genes and alternative culture collections repositories for the other DNA targets are noted in Table S8.3. Branch support is recorded at nodes as a percentage of 1,000 bootstrap iterations. *CCUG 48898 is the type strain of "*M. massiliense*".

Isolate D16R27 was selected to perform DNA–DNA hybridization (DDH) experiments. High-molecular weight DNA was prepared from 2 g cell mass using the protocol described by Pitcher *et al*. (1989) with modifications (Pitcher, Saunders et al. 1989). After centrifugation, cells were inactivated at 90°C for 30 minutes and resuspended in 3 mL of lysis buffer containing 200 µg RNAse ml^{-1}, 25 mg fresh lysozyme ml^{-1} and 100U mutanolysine ml^{-1}. The suspensions were incubated overnight at 37°C. Chloroform/isoamyl alcohol extraction, RNase treatment and ethanol precipitation were performed as described by Marmur (Mamur 1961). DDH was performed with photobiotin-labelled probes in microplate wells, as described by Ezaki *et al*. (1989), using HTS7000 Bio Assay Reader (Perkin-Elmer) for fluorescence measurements (Ezaki, Hashimoto et al. 1989). The hybridization temperature was 50^{o}C, calculated on the basis of the DNA G+C contents of the isolates and type strains (Mesbah and Whitman 1989). DNA G+C content of isolates D16R27 and D16Q19 was 64.2 mol%. The G+C content of *M. abscessus* subsp. *abscessus* ATCC 19977^{T}, *M. chelonae* ATCC 35752^{T}, *M. immunogenum* ATCC 700505^{T}, *M. salmoniphilum* ATCC 13758^{T} and *M. franklinii* DSM 45524^{T} was 64.0 mol%, 64.1 mol%, 64.2 mol%, 64.3 mol% and 64.1 mol%, respectively. These values are consistent with the G+C contents of this genus, between 59 mol% and 66 mol% (Devulder, Perouse de Montclos et al. 2005). DNA-DNA hybridization values are presented as averages of

reciprocal experiments, performed in quadruplicate hybridization reactions (Table 8.2). The average DNA-DNA hybridization value between D16R27 and *M. franklinii* DSM 45524^T was higher than the cutoff value of 70% and confirmed that both belong to the same species. The average hybridization values of D16R27 with *M. abscessus* subsp. *abscessus* ATCC19977^T, *M. chelonae* ATCC 35752^T, *M. immunogenum* ATCC 700505^T, *M. salmoniphilum* ATCC 13758^T were all below 70%, further confirming that the isolate D16R27 represents a distinct species of the *M. chelonae-M. abscessus* group (Table 8.2).

Table 8.2. DNA-DNA hybridization values of D16R27 against the type strains of *M. chelonae-M. abscessus* group. Reciprocal experiments were performed in quadruplicate hybridization reactions.

Species	Strain	DNA-DNA relatedness (%)*	Reciprocal values
M. franklinii	DSM 45524^T	78.5 (2.5)	76;81
M. abscessus subsp. *abscessus*	ATCC 19977^T	50.5 (4.5)	46;55
M. chelonae	ATCC 35752^T	55 (8)	47;63
M. immunogenum	ATCC 700505^T	45.5 (4.5)	41;50
M. salmoniphilum	ATCC 13758^T	51 (10)	41;61

*Values are mean with standard deviation

In conclusion, phenotypic and genotypic characteristics of isolates D16R27, D16Q19 and *M. franklinii* DSM 45524^T indicate that these isolates belong to the *M. chelonae-M. abscessus* group. In addition, several results clearly indicate that D16R27, D16Q19 and *M. franklinii* DSM 45524^T form a homogeneous group separated from other members of *M. chelonae-M. abscessus*. Therefore, we propose to formally name this new member of the *M. chelonae-M. abscessus* group *M. franklinii* sp. nov., which was originally described but not validly named by Simmon & Brown-Elliott *et al.* (Simmon, Brown-Elliott et al. 2011).

Description of *M. franklinii* sp. nov.

M. franklinii (frank.li′ni.i I, N.L. masc. gen. n. *franklinii* of Franklin, pertaining to Benjamin Franklin, famous United States of America statesman and scientist from Pennsylvania where the first isolates originated).

The cells are acid-fast bacilli and visible growth on solid media requires 3-5 days of incubation at 28^{o}C. Colonies are nonpigmented and growth occurs in the presence of 5% NaCl, picric acid, PNB and nitrite. Growth is not observed when mannitol or inositol is used as single-source of carbon. The type strain *M. franklinii*

DSM 45524^T does not grow when citrate is used as single-source of carbon. Negative reactions are observed for nitrate reduction and Tween 80 hydrolysis. Conventional biochemical tests cannot distinguish *M. franklinii* from other members of *M. chelonae-M. abscessus* group and should not be used as confirmatory tests for identification of this species. *M. franklinii* isolates were susceptible to clarithromycin at 3 days. Eight of the original 25 isolates had clarithromycin MICs at ≤2 at 14 days. However, these data were not previously published. Isolates were susceptible to amikacin, ciprofloxacin, moxifloxacin, doxycycline, and minocycline. Tobramycin and cefoxitin had variable susceptibility (see Table 1). Partial sequencing of *rpoB* gene can distinguish *M. franklinii* from other members of *M. chelonae-M. abscessus* group – *M. franklinii* has 726 bp fragment length while the corresponding sequences of the type strains of *M. chelonae-M. abscessus* group have a 711 bp fragment length. The PRA-*hsp65* pattern that characterizes *M. franklinii* is BstEII [bp] (320, 130) and HaeIII [bp] (200, 70, 60, 55), these fragment sizes are closely related to the pattern of *M. immunogenum* type 2.

The type strain is DSM 45524^T (= ATCC BAA-2149^T).

Acknowledgements

This study received financial support from Fundação de Amparo à Pesquisa do Estado de São Paulo (www.fapesp.br) (FAPESP) (grant 2011/18326-4). C.L.N. received a fellowship from FAPESP (2012/13763-0).

Supplementary material

Table S8.1. Comparison of phenotypic and biochemical characteristics of the isolates and the type strains included in this study.

Isolates/strains: 1, D16Q19; 2, D16R27; 3, *M. franklinii* DSM 45524^T; 4, M. *abscessus subsp. abscessus* ATCC 19977^T; 5, *M. abscessus subsp. bolletii* CCUG 50184^T; 6, *M. chelonae* ATCC 35752^T; 7, *M. immunogenum* ATCC 700505^T; 8, *M. salmoniphilum* ATCC 13758^T

Phenotypic and biochemical tests	Isolates and strains							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Colony morphology	RG	SM	SM	SM	SM	SM	RG	SM
Pigmentation	absent	absent	absent	absent	absent	absent	absent	absent
Growth in the presence of:								
Picric acid	+	+	+	+	+	+	+	+
PNB*	+	+	+	+	+	+	+	+
Nitrite	+	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+	+
Nitrate reduction	-	-	-	-	-	-	-	-
Tween hydrolysis	-	-	-	-	-	-	-	-
single-source carbon utilization of:								
mannitol	-	-	-	-	-	-	+	-
inositol	-	-	-	-	-	-	-	-
citrate	+	+	-	-	-	+	+	-

*PNB, *p*-aminobenzoic acid; SM, smooth; RG, rough

Table S8.2. Primers used in genotypic characterization of strains and isolates included in this study

Gene	Primers	Sequence 5´-3´	References
16S rRNA	16S-27F*	AGAGTTTGATCCTGGCTCAG	(Harmsen, Dostal et al. 2003)
16S rRNA	800F	ATTAGATACCCTGGTAG	(Adekambi and Drancourt 2004b)
16S rRNA	16S-907R*	CCGTCAATTCCTTTGAGTTT	(Harmsen, Dostal et al. 2003)
16S rRNA	16R-1492*	TACGGCTACCTTGTTACGACTT	(Gomila, Ramirez et al. 2007)
rpoB	MycoF	GGCAAGGTCACCCCGAAGGG	(Adekambi, Colson et al. 2003)
rpoB	MycoR	AGCGGCTGCTGGGTGATCATC	(Adekambi, Colson et al. 2003)
hsp65	Tb11	ACCAACGATGGTGTGTCCAT	(Telenti, Marchesi et al. 1993)
hsp65	Tb12	CTTGTCGAACCGCATACCCT	(Telenti, Marchesi et al. 1993)
ITS	Sp1	ACCTCCTTTCTAAGGAGCACC	(Roth, Reischl et al. 2000)
ITS	Sp2	GATGCTCGCAACCACTATCCA	(Roth, Reischl et al. 2000)

*Primers were modified based on the *M. abscessus* subsp. *abscessus* ATCC 19977^T genome

Table S8.3. Percent identity of the partial sequences of isolates D16Q19 and D16R27 and the sequences of the *M. chelonae-M. abscessus* group type strains.

Only the regions between the primers used for amplification and present in all sequences were considered for comparison, excluding indels.

Gene	Type strain*	Accession number	D16Q19 = D16R27
16S rRNA (1384 bp)#	*M. abscessus* subsp. *abscessus* CIP 104536^T	AY457071	99.71%
	M. abscessus subsp. *bolletii* CIP 108541^T	AY859681	99.71%
	M. abscessus subsp. *bolletii* CCUG 48898	NR043002	99.71%
	M. chelonae CIP 104535^T	AY457072	100.00%
	M. immunogenum DSM 45595^T	HE654001	99.35%
	M. salmoniphilum ATCC 13758^T	NR_043989	99.64%
	M. franklinii DSM 45524^T	HQ153090	100.00%
hsp65 (401 pb)	*M. abscessus* subsp. *abscessus* CIP 104536 ^{T}	AY458075	92.77%
	M. abscessus subsp. *bolletii* CIP 108541^T	AY859675	92.77%
	M. abscessus subsp. *bolletii* CCUG 48898	AY596465	93.02%
	M. chelonae CIP 104535^T	AY458074	96.01%
	M. immunogenum CIP 106684^T	AY458081	98.25%
	M. salmoniphilum ATCC 13758^T	DQ866777	97.26%
	M. franklinii DSM 45524^T	KM392059	99.75%
rpoB (711 bp)§	*M. abscessus* subsp. *abscessus* CIP 104536 ^{T}	AY147164	93.95%
	M. abscessus subsp. *bolletii* CIP 108541^T	AY859692	95.22%
	M. abscessus subsp. *bolletii* CIP 108297	EU254721	95.92%
	M. chelonae CIP 104535^T	AY147163	96.77%
	M. immunogenum CIP 106684^T	AY262739	96.48%
	M. salmoniphilum ATCC 13758^T	KM392058	96.20%
	M. franklinii DSM 45524^T	KM392056	98.59%
ITS (214 bp)¶	*M. abscessus* subsp. *abscessus* CIP 104536^T	AY593976	93.46%
	M. abscessus subsp. *bolletii* CCUG 50184^T	GU143888.2	93.46%
	M. abscessus subsp. *bolletii* CCUG 48898	AY593978	92.99%
	M. chelonae ATCC 35752^T	AY498739	96.26%
	M. immunogenum CIP 106684^T	AY593977	89.72%
	M. salmoniphilum ATCC 13758^T	DQ866768	97.66%
	M. franklinii DSM 45524^T	HQ153093	99.07%

* strain designations according to the deposits in culture collections and GenBank sequence database. CCUG 48898 and CIP 108297 are the type strain of *"M. massiliense"*

The 16S rRNA gene partial sequence of *M. immunogenum* DSM 45595^T has 1385 bp due to a 1 bp insertion after nucleotide 1354.

§ The *rpoB* partial sequences of D16R27, D16Q19 and *M. franklinii* DSM 45524^T have 726 bp and the corresponding sequences of the other type strains of the *M. chelonae-M. abscessus* group have 711 bp. This difference is due to two insertions in the *M. franklinii* isolates, one with 6 bp, between nucleotides 409 and 410 and the other with 9 bp, between nucleotides 424 and 425 of the 711 bp sequence.

¶ The ITS sequences of D16R27, D16Q19, *M. franklinii* DSM 45524^T and *M. salmoniphilum* ATCC 13758^T have 214 bp, those of *M. abscessus* subsp. *abscessus* ATCC 19977^T, *M. abscessus* subsp. *bolletii* CCUG 50184^T and *M. chelonae* ATCC 35752^T have 215 bp (1 bp insertion after nucleotide 20), that of "*M. massiliense"* CCUG 48898 has 216 bp and that of *M. immunogenum* ATCC 700505^T has 225 bp (10 bp insertion between nucleotides 90 and 91)

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9 Description of *Mycobacterium saopaulense* sp. nov., a rapidly growing mycobacterium closely related with members of the *Mycobacterium chelonae-M. abscessus* group

Redraft from: **Nogueira CL**, Whipps CM, Matsumoto CK, Chimara E, Droz S, Tortoli E, de Freitas D, Cnockaert M, Palomino JC, Martin A, Vandamme P, Leão SC. Description of *Mycobacterium saopaulense* sp. nov., a rapidly growing mycobacterium closely related with members of the Mycobacterium chelonae-M. abscessus group. International Journal of Systematics and Evolutionary Microbiology 65: 4403-4409, 2015.

Abstract

Five isolates of non-pigmented rapidly growing mycobacteria were isolated from three patients and, in an earlier study, from zebrafish. Phenotypic and molecular tests confirmed that these isolates belong to the *Mycobacterium chelonae-Mycobacterium abscessus* group, but they could not be confidently assigned to any known species of this group. Phenotypic analysis and biochemical tests were not helpful for distinguishing these isolates from other members of the *M. chelonae-M. abscessus* group. They presented higher drug resistance in comparison with other members of the group, showing susceptibility to clarithromycin only. The five isolates showed a unique PCR restriction analysis pattern of the *hsp*65 gene, 100% identity in 16S rRNA and *hsp65* sequences and 1-2 nucleotide differences in *rpoB* and in the internal transcribed spacer (ITS). Phylogenetic analysis of a concatenated data set including 16S rRNA, *hsp65*, and *rpoB* gene sequences from type strains of more closely related species placed these isolates together, as a distinct lineage from previously described species suggesting a sister relationship to a group consisting of *M. chelonae*, *M. salmoniphilum*, and *M. immunogenum*. DNA-DNA hybridization values >70% confirmed that the five isolates belong to the same species, while the values <70% between one of them and the type strains of *M. chelonae* and *M. abscessus* confirmed their belonging to a distinct species. The polyphasic characterization of these isolates, supported by DNA-DNA hybridization results, demonstrated that they share characteristics with *M. chelonae-M. abscessus* members, but constitute a different species, for which the name *Mycobacterium*

saopaulense sp. nov. is proposed. The type strain is EPM 10906^T (CCUG 66554^T = LMG 28586^T = INCQS 0733^T).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA sequences are KM973037 (EPM 10906^T), KM973036 (EPM 10695), KM973038 (IAL 3785), DQ866774 (JAN1) and DQ866775 (JAN2); for the *hsp*65 are KM973026 (EPM 10906^T), KM973025 (EPM 10695), KM973027 (IAL 3785), DQ866786 (JAN1) and DQ866787 (JAN2); for the *rpoB* are KM973029 (EPM 10906^T), KM973028 (EPM 10695), KM973030 (IAL 3785), KM973031 (JAN1) and KM973032 (JAN2); and for the ITS are KM973034 (EPM 10906^T), KM973033 (EPM 10695), KM973035 (IAL 3785) and DQ866774 (JAN1) and DQ866775 (JAN2).

Nontuberculous mycobacteria are ubiquitous environmental organisms and several species can cause opportunistic infections in humans, in particular the members of the *Mycobacterium chelonae-Mycobacterium abscessus* group. It comprises closely related rapidly growing mycobacteria that can cause a broad spectrum of infections mainly affecting lung, skin and soft tissue (Wallace Jr, Swenson et al. 1983, Wallace, Swenson et al. 1983, Simmon, Brown-Elliott et al. 2011). The ubiquitous distribution of these organisms facilitates the contamination of medical equipment and solutions that, associated with the growing number of therapeutic interventions, generate nosocomial infections and outbreaks representing a serious public health problem in some settings (Tortoli 2009, Leao, Viana-Niero et al. 2010).

Until recently, the *M. chelonae-M. abscessus* group was composed of *M. chelonae*, *M. abscessus* (Kusunoki and Ezaki 1992), *Mycobacterium immunogenum* (Wilson, Steingrube et al. 2001), *Mycobacterium massiliense* (Adekambi, Reynaud-Gaubert et al. 2004a, Adekambi, Berger et al. 2006), *Mycobacterium bolletii* (Adekambi, Berger et al. 2006), and *Mycobacterium salmoniphilum* (Whipps, Butler et al. 2007). Taxonomic changes have been proposed and *M. abscessus*, *M. massiliense* and *M. bolletii* have been assigned to a single species (*M. abscessus*) with two subspecies, *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp.

bolletii, the latter including those isolates previously identified as *M. massiliense* and *M. bolletii* (Leao, Tortoli et al. 2009, Leao, Tortoli et al. 2011). Two novel members of this group have been recently described, *Mycobacterium franklinii* (Simmon, Brown-Elliott et al. 2011, Lourenco Nogueira, Simmon et al. 2015) and "*Mycobacterium fukienense*" (Zhang, Li et al. 2013).

The aim of the present study was to define the taxonomic position of five mycobacterial isolates (EPM 10906^T, EPM 10695, IAL 3785, JAN1 and JAN2) without conclusive species assignments, which share a PCR Restriction Analyses (PRA) profile of the *hsp*65 gene not present in the PRASITE database (http://app.chuv.ch/prasite/index.html). Our data indicate that these five isolates belong to a single taxon and represent a novel species of the *M. chelonae-M.abscessus* group. We therefore propose the name *Mycobacterium saopaulense* sp. nov., with the type strain EPM 10906^T.

The first two isolates (EPM 10906^T and EPM 10695) were obtained in 1999 from corneal specimens of two patients with infectious crystalline keratopathy after LASIK surgery (laser-assisted *in situ* keratomileusis) performed in the same ophthalmological clinic, in São Paulo city (Brazil). These isolates were first misidentified as *M. chelonae* (Alvarenga, Freitas et al. 2002) and subsequently as *M. abscessus* (Sampaio, Junior et al. 2006), on the basis of PRA of the *hsp65* gene. Typing of these isolates by pulsed field gel electrophoresis (PFGE) using a protocol previously described (Matsumoto, Chimara et al. 2011), revealed that they share indistinguishable patterns and thus might belong to a single strain (Fig. 9.1). The third isolate (IAL 3785) was obtained in 2007 from a cervical abscess in the city of Ribeirão Preto, São Paulo (Brazil). The other two isolates (JAN1 and JAN2) were isolated from zebrafish (*Danio rerio*) (Kent, Whipps et al. 2004) and initially categorized as *M. chelonae*. Using greater taxon sampling in a later phylogenetic analysis, these strains were recognized as phylogenetically distinct (Whipps, Butler et al. 2007), but they were not described as a new species at the time. JAN1 and JAN2 share highly similar PFGE patterns differing in a single band and were isolated from zebrafish at the same research facility about two months apart in 2003 (Kent, Whipps et al. 2004). Considering this they could represent a single strain.

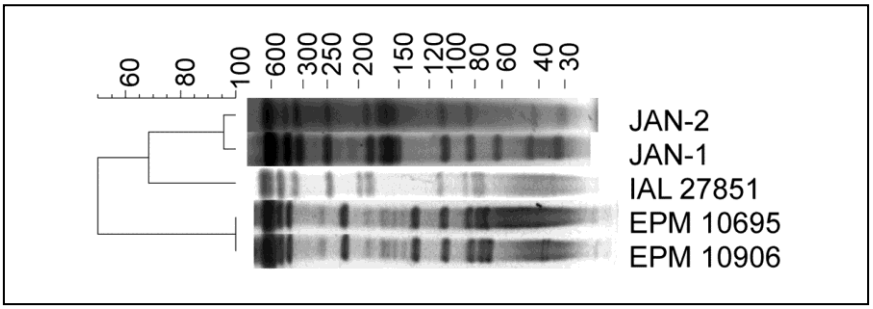


Figure 9.1. Pulsed-field gel electrophoresis (PFGE) patterns of DraI digested DNA of the five isolates studied in this work. PFGE images were analyzed with the BioNumerics program v. 7.1 (Applied Maths, Sint-Martens-Latem, Belgium). The band-based Dice unweighted-pair group method using average linkages was used to prepare a dendrogram of PFGE profiles, based on 1.5% optimization and position tolerance.

We investigated the classification of these five isolates, comprising three different strains, using a polyphasic approach that included microscopic and macroscopic morphological examination, cultural and biochemical tests, drug susceptibility testing, HPLC analysis of cell wall mycolic acids, PRA, sequencing of three housekeeping genes and DNA-DNA hybridization. The results were compared with those displayed by the type strains of *M. abscessus* subsp. *abscessus* ATCC 19977^T, *M. abscessus* subsp. *bolletii* CCUG 50184^T, *M. chelonae* ATCC 35752^T, *M. immunogenum* ATCC 700505^T, *M. salmoniphilum* ATCC 13758^T and *M. franklinii* DSM 45524^T.

Cultures were grown on solid media, Löwenstein-Jensen (LJ), Middlebrook 7H10 supplemented with OACD (oleic acid, albumin, dextrose and catalase) and Luria-Bertani agar, and in liquid Muller-Hinton medium or Luria-Bertani broth with 1% Tween 80 at 28^{o}C–30^{o}C for 5 days. Microscopic examination of colony smears by Ziehl-Neelsen staining confirmed that they are acid-fast bacilli. Analysis of pigment production, single-source carbon utilization (mannitol, inositol and citrate), growth at 26^{o}C and 37^{o}C and tolerance to 5% NaCl, 0.2% picric acid, para-nitrobenzoic acid (PNB) and nitrite were performed on 7H10-OADC and LJ. Nitrate reduction, Tween 80 hydrolysis and arylsulfatase production were also examined. All tests were performed as described in standard protocols for biochemical identification of mycobacteria (Tsukamura 1984, Kent and Kubica 1985, Leão, Martin et al. 2004). The five isolates exhibited indistinguishable phenotypic and biochemical

characteristics, which are listed in Table S9.1. These cultural and biochemical tests were not helpful for distinguishing these isolates from other members of the *M. chelonae-M. abscessus* group.

Antimicrobial drug-susceptibility testing was performed using a microdilution method in cation-supplemented Mueller–Hinton broth, according to the recommendations of the Clinical and Laboratory Standards Institute for rapidly growing mycobacteria (CLSI 2011). Amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxycycline, minocycline, moxifloxacin and tobramycin were tested. The five isolates were more drug resistant than the other members of *M. chelonae-M. abscessus* group, showing susceptibility only to clarithromycin at 3 and 14 days incubation. They were resistant to doxycycline, cefoxitin and tobramycin and resistant or intermediate to amikacin, ciprofloxacin, minocycline and moxifloxacin (Table 9.1). These results are consistent with susceptibility testing previously conducted for strain JAN1 (Chang and Whipps 2015). The drug resistance profile of *M. saopaulense* sp. nov. highlights the importance of its correct identification for patient management.

Table 9.1. Antimicrobial susceptibility results for isolates and type strains included in this study

Isolates/strains: 1, EPM 10695; 2, EPM 10906^T; 3, IAL 3785; 4, JAN1; 5, JAN2; 6, *M. abscessus* subsp. *abscessus* ATCC 19977^T; 7, *M. abscessus* subsp. *bolletii* CCUG 50184^T; 8, *M. chelonae* ATCC 35752^T; 9, *M. immunogenum* ATCC 700505^T; 10, *M. salmoniphilum* ATCC 13758^T; 11, *M. franklinii* DSM 45524^T.

Drugs	MIC (µg ml^{-1})										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Amikacin	32 I	128 R	32 I	32 I	32 I	8 S	16 S	8 S	≤ 4 S	16 S	≤ 4 S
Ciprofloxacin	2 I	2 I	4 R	4 R	4 R	4 R	8 R	0.5 S	1 S	1 S	0.5 S
Clarithromycin (3 days)	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S
Clarithromycin (14 days)	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S	>64 R	>64 R	≤ 0.5 S	≤ 0.5 S	ND	≤ 0.5 S
Doxycycline	>32 R	>32 R	>32 R	>32 R	>32 R	>32 R	>32 R	4 I	>32 R	>32 R	≤ 0.25 S
Cefoxitin	512 R	>512 R	512 R	>512 R	512 R	64 I	32 I	512 R	256 R	512 R	16 S
Tobramycin	16 R	32 R	16 R	8 R	16 R	8 R	16 R	2 S	4 I	4 I	2 S
Minocycline	32 R	16 R	4 I	16 R	8 R	8 R	16 R	2 I	8 R	2 I	≤ 0.25 S
Moxifloxacin	2 I	8 R	4 R	4 R	2 I	8 R	8 R	≤ 0.25 S	1 S	2 I	1 S

(R) resistant, (I) intermediate, S (susceptible) – criteria for rapidly growing mycobacteria established by CLSI (2011)
(ND) not done

For HPLC analysis of cell wall mycolic acids two strains of the panel characterized here were selected (the proposed type strain EPM 10906^T and JAN1) and three reference strains belonging to the closely related *M. chelonae-M. abscessus* group (ATCC 19977^T, CCUG 48898^T and CCUG 50184^T). The cells of the strains above, grown in culture on Middlebrook 7H10 agar, were saponified, extracted and derivatized as recommended by the Sherlock Mycobacteria Identification System (SMIS; MIDI) and separated using a gradient of methanol and isopropanol. All the strains analyzed produced nearly identical HPLC patterns characterized by two late emerging clusters of peaks (Figure 9.2). The Sherlock software (version Myco 1.0) identified all of them as *M. chelonae-M. abscessus* with very high similarity indexes (range 0.802-0.899). The low discriminatory power of HPLC analysis in differentiating most rapidly growing mycobacterial species (Tortoli 2003) is therefore confirmed for the proposed new species, with this approach being unsuitable to go further in the assignation to the *M. chelonae-M. abscessus* group.

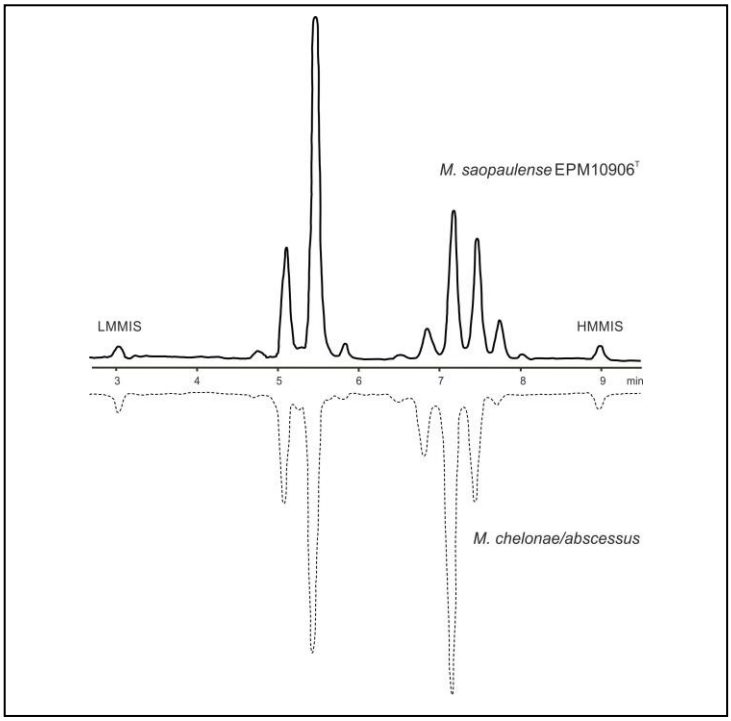


Figure 9.2. Representative mycolic acid pattern of *Mycobacterium saopaulense* sp. nov. EPM10906^T paired with the reference profile of *Mycobacterium chelonae-Mycobacterium abscessus* (Sherlock database). LMMIS, low molecular mass internal standard; HMMIS, high molecular mass internal standard.

GenoType® *Mycobacterium* (Hain Lifescience, Nehren Germany), a commercial DNA strip assay for mycobacteria identification, was performed according to manufacturer's instructions. Using the GenoType® CM strip, the isolates showed the *M. chelonae* profile (hybridization with probes 5 and 10). GenoType® AS only identified the isolates at level of *Mycobacterium* species.

For molecular identification, the PRA of the *hsp*65 gene and of ITS and the partial sequencing of the small subunit (16S) rRNA gene, *rpoB*, *hsp*65 and ITS were performed. The DNA was prepared by boiling one loop full of bacteria for 10 min in 300 µL of TET (10 mM Tris, 1 mM EDTA, 1% Triton X-100 [pH 8.0]) followed by centrifugation at 14,000 *g* for 2 min. For PRA-*hsp*65, a 441-bp fragment of the *hsp*65 gene was amplified using primers Tb11 and Tb12 (Table S9.2), and the amplicon was digested in two separate tubes with BstEII and HaeIII restriction enzymes (Telenti, Marchesi et al. 1993). For PRA-ITS, amplicons generated with primers Sp1 and Sp2 (Table S9.2) were digested with TaqI restriction enzyme (Roth, Reischl et al. 2000). PRA-*hsp*65 and PRA-ITS digestion products were visualized in 3% agarose gels stained with ethidium bromide after electrophoresis, using 50-bp ladder as the molecular size standard. The restriction fragment sizes were estimated using the BioNumerics program version 7.1 (Applied Maths, Sint-Martens-Latem, Belgium) and compared to the patterns included in the PRASITE for PRA-*hsp*65 and published by Roth *et al.* (2000) for PRA-ITS (Roth, Reischl et al. 2000). The five isolates showed identical PRA-*hsp*65 patterns – BstEII [bp] (235, 210) and HaeIII [bp] (145, 60, 50), which differs from patterns of other *M. chelonae-M. abscessus* members. This profile was not registered in the PRASITE database. They showed the same PRA-ITS pattern – TaqI [bp] (225, 30), which is common to *M. abscessus* and *M. franklinii*. Therefore, only PRA-*hsp*65 was useful for differentiation of *M. saopaulense* sp. nov.

The primers used for PCR amplification and partial sequencing of 16S rRNA, *hsp*65, *rpoB* and ITS are listed in Table S9.2. PCR products were purified using QIAquick PCR purification Kit (Qiagen, Germany). Dideoxy sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and run in ABI PRISM 3100 DNA Analyzer (Applied Biosystems). Using ClustalW multiple alignment (BioEdit version 7.2.5), the obtained sequences of isolates EPM 10906^{T}, EPM 10695, IAL 3785, JAN1 and JAN2 were aligned and the percentages of sequence identity were calculated after alignment. The five isolates

shared 100% identity in the partial *hsp*65 and 16S rRNA sequences and had 1-2 nucleotide differences in the partial sequences of *rpoB* and ITS. The high similarity of the 16S rRNA to *M. chelonae* had been previously recognized by Kent *et al*. (Kent, Whipps et al. 2004) who assigned the two isolates investigated to such species. Greater differences in other genes (*rpoB*, *hsp65*, ITS) and in the PRA-*hsp65* pattern subsequently revealed that these isolates are distinct (Sampaio, Junior et al. 2006, Whipps, Butler et al. 2007), and this is supported by the data presented here.

Previous phylogenetic analyses (Sampaio, Junior et al. 2006, Whipps, Butler et al. 2007) of individual genes had shown sister relationships of JAN1 and JAN2 isolates to different species. In the tree from *hsp65* tree, these isolates were sister to *M. abscessus*. In the tree from *rpoB* they showed a sister relationship to the entire *M. chelonae-M. abscessus* group. In the tree from ITS a sister relationship between *M. saopaulense* sp. nov. and *M. abscessus* emerged. Analysis of 16S rRNA alone revealed that sequences from the five isolates, *M. abscessus*, and *M. chelonae* are intermixed (Figure S9.1). Thus, 16S rRNA alone was not sufficient to resolve these relationships and other genes yielded conflicting results. For this study, we chose to analyze a concatenated data set using 16S rRNA, *hsp65*, and *rpoB* from type species (Table S9.3). Sequences were aligned with Clustal X version 1.8 (Thompson, Gibson et al. 1997) with default parameters (penalty for gap opening = 10, gap extension = 0.2). A maximum likelihood analysis was performed using PhyML (Guindon and Gascuel 2003) and bootstrap confidence values were calculated with 100 replicates. The resulting tree was edited and annotated in Adobe Illustrator (Adobe Systems Inc. San Jose, California). The ITS region was excluded from the final analysis because homologous sites were difficult to confidently identify from outgroup taxa; however, when ITS data are included (unalignable data treated as missing) tree topology was unaltered (data not shown). In either analysis, the five isolates showed a sister relationship to a clade made up of 4 species (*M. chelonae*, *M. salmoniphilum*, *M. immunogenum*, and *M. franklinii*) (Figure 9.3).

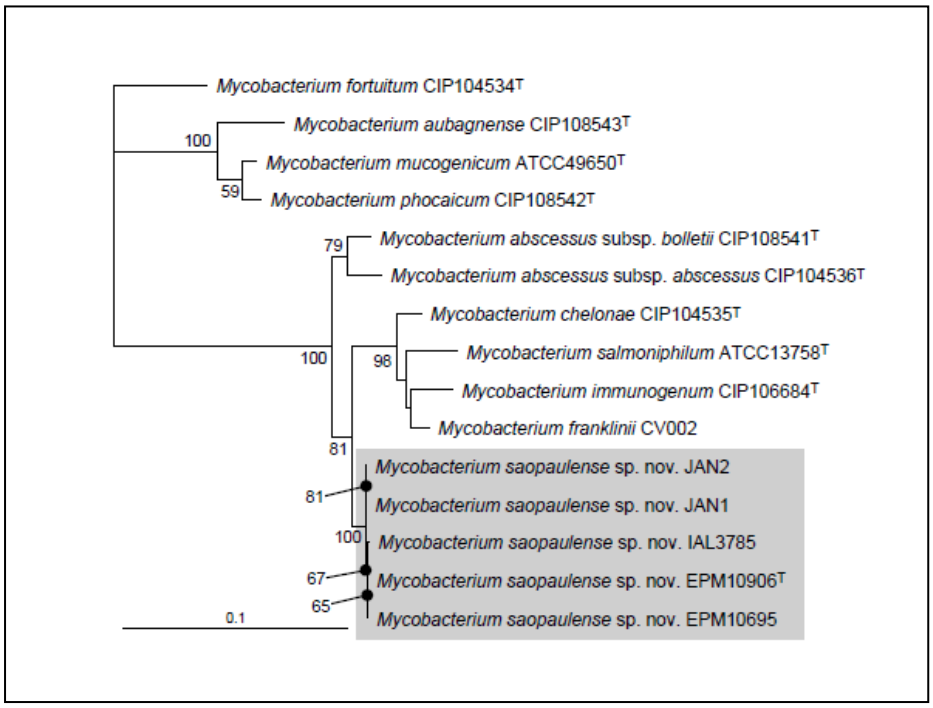


Figure 9.3. Estimate of phylogeny of *Mycobacterium saopaulense* sp. nov. and other closely related rapid growers based on maximum likelihood analysis of concatenated data set of 16S rRNA, *hsp65*, and *rpoB*. Bootstrap support values greater than 50% are shown at nodes. Genbank accession of the 16S rRNA, *hsp65* and *rpoB* genes are shown in Table S3.

These plus the five isolates are sister to *M. abscessus*. This concatenated data present the phylogenetic hypothesis that the five isolates are sister to *M. chelonae* versus *M. abscessus* as suggested by individual gene trees from *hsp65* and ITS (Whipps, Butler et al. 2007). Regardless of the precise sister relationship, all analyses support inclusion in the *M. chelonae-M. abscessus* group, and a phylogenetic position for the five isolates that is distinct from all other nominal species.

For DDH, high-molecular weight DNA was prepared from 2 g of cell mass using the protocol described by Pitcher *et al*. (1989) with modifications (Pitcher, Saunders et al. 1989). Bacterial cells were centrifuged, inactivated at 90°C for 30 minutes and resuspended in 3 mL of lysis buffer containing 200 µg RNase ml^{-1}, 25 mg fresh lysozyme ml^{-1} and 100U mutanolysine ml^{-1}. The suspensions were incubated overnight at 37°C and the DNA was extracted with chloroform/isoamyl alcohol, treated with RNase and precipitated with ethanol as described by Mamur (1961). DDH was performed as described by Ezaki *et al*. (1989), using photobiotin-

labelled probes in microplate wells (Ezaki, Hashimoto et al. 1989). Fluorescence was measured in HTS7000 Bio Assay Reader (Perkin-Elmer). DNA-DNA hybridization values are presented as averages of reciprocal experiments, performed in quadruplicate hybridization reactions. The DNA G+C content, estimated as described by Mesbah and Whitman (1989), was 64.6 mol% (EPM 10906^T), 64.8 mol% (EPM 10695), 64.7 mol% (IAL 3785), 64.5 mol% (JAN1) and 64.7 mol% (JAN2). These values were used for calculation of the 50^{o}C hybridization temperature. They are consistent with the G+C contents of this genus, between 59 mol% and 66 mol% (Devulder, Perouse de Montclos et al. 2005). DDH experiments performed with the five isolates yielded hybridization values above 70% (data not shown), confirming that they belong to the same species. EPM 10906^T was selected to perform DDH reciprocal experiments with the type strains of the *M. chelonae-M. abscessus* group. All values were below 70%, confirming that EPM 10906^T and the other four isolates belong to a distinct species of the *M. chelonae-M. abscessus* group (Table 9.2).

Table 9.2. DNA-DNA hybridization values of EPM 10906^T against the type strains of *M. chelonae-M. abscessus* group. Reciprocal experiments were performed in quadruplicate hybridization reactions.

Species	Strain	DNA-DNA relatedness (%)*	Reciprocal values
M. abscessus subsp. *abscessus*	ATCC 19977^T	41.5 (2.5)	39;44
M. abscessus subsp. *bolletii*	CCUG 50184^T	40 (1)	39;41
M. chelonae	ATCC 35752^T	47 (5)	42;52
M. immunogenum	ATCC 700505^T	40 (7)	33;47
M. salmoniphilum	ATCC 13758^T	44.5 (1.5)	43;46
M. franklinii	DSM 45524^T	51 (4)	47;55

*Values are mean with standard deviation

In conclusion, phenotypic and genotypic tests indicated that EPM 10906^T, EPM 10695, IAL 3785, JAN1 and JAN2 belong to the *M. chelonae-M. abscessus* group. In addition, several results clearly indicated that they form a uniform group separated from the other members of this group. Therefore, we propose to classify these isolates as a new member in the *M. chelonae-M. abscessus* group, with the name *M. saopaulense* sp. nov.

Description of *Mycobacterium saopaulense* sp. nov.

Mycobacterium saopaulense (sa.o.paul.en'se. (N.L. neut. adj. *saopaulense*, of or pertaining to the Brazilian state of São Paulo, where the first strains were isolated).

The cells are acid-fast bacilli and visible growth on solid media requires 3-5 days at 28^{o}C. Colonies are nonpigmented and smooth. After some days the medium often acquires a brown color. Growth occurs in the presence of 5% NaCl, picric acid, PNB, nitrite. Growth is observed in the presence of citrate as single-source of carbon but not of mannitol or inositol. Negative reactions are observed for nitrate reduction and Tween 80 hydrolysis. Conventional biochemical testing cannot distinguish *M. saopaulense* sp. nov. from other members of *M. chelonae-M. abscessus* group. The antimicrobial pattern is characterized by susceptibility to clarithromycin and resistance to doxycycline, tobramycin and cefoxitin. Variable results, intermediate or resistant, were obtained with amikacin, ciprofloxacin, minocycline and moxifloxacin. The mycolic acid profile is similar to that of *M. chelonae-M. abscessus* by HPLC analysis. Genotype® CM shares the same profile of *M. chelonae*. The partial sequencing of *rpoB* and *hsp*65 genes can distinguish *M. saopaulense* sp. nov. from other members of *M. chelonae-M. abscessus* group. The PRA-*hsp65* pattern that characterizes *M. saopaulense* sp. nov. is BstEII [bp] (235, 210) and HaeIII [bp] (145, 60, 50). The type strain of *M. saopaulense* sp. nov. is EPM 10906^{T} (CCUG 66554^{T} = LMG 28586^{T} = INCQS 0733^{T}).

Acknowledgements

This study received financial support from Fundação de Amparo à Pesquisa do Estado de São Paulo (www.fapesp.br) (FAPESP) (grant 2011/18326-4). C.L.N. and C.K.M. received fellowships from FAPESP (2012/13763-0 and 2013/16018-6). This work has been partially supported by International Cooperation UAM-Banco Santander and Latin America (CEAL-UAM). Contributions to this work by C.M.W. were funded in part by the Office of Research Infrastructure Programs of the National Institutes of Health (NIH) under award number R24OD010998. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Supplementary material

Table S9.1. Comparison of phenotypic and biochemical characteristics of the isolates and the type strains included in this study.

Isolates/strains: 1, EPM10906; 2, EPM10695; 3, IAL 3785; 4, JAN1; 5, JAN2, 6, *M. abscessus subsp. abscessus* ATCC 19977^T; 7, *M. abscessus subsp. bolletii* CCUG 50184^T; 8, *M. chelonae* ATCC 35752^T; 9, *M. immunogenum* ATCC 700505^T; 10, *M. salmoniphilum* ATCC 13758^T; 11, *M. franklinii* DSM 45524^T

Characteristics	1	2	3	4	5	6	7	8	9	10	11
Colony morphology	SM	SM	SM	SM	SM	SM	SM	SM	RG	SM	SM
Pigmentation	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc
Growth in the presence of:											
Picric acid	+	+	+	+	+	+	+	+	+	+	+
PNB*	+	+	+	+	+	+	+	+	+	+	+
Nitrite	+	+	+	+	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-
Tween 80 hydrolysis	-	-	-	-	-	-	-	-	-	-	-
single-source carbon utilization of:											
mannitol	-	-	-	-	-	-	-	-	+	-	-
inositol	-	-	-	-	-	-	-	-	-	-	-
citrate	+	+	+	+	+	-	-	+	+	-	-

*PNB, *p*-aminobenzoic acid; SM, smooth; RG, rough; nc, nonchromogenic

Table S9.2. Primers used in genotypic characterization of strains and isolates included in this study

Gene	Primers	Sequence 5´-3´	References
16S rRNA	16S-27F*#	AGAGTTTGATCCTGGCTCAG	(Harmsen, Dostal et al. 2003)
16S rRNA	800F*	ATTAGATACCCTGGTAG	(Adekambi and Drancourt 2004b)
16S rRNA	16S-907R*#	CCGTCAATTCCTTTGAGTTT	(Harmsen, Dostal et al. 2003)
16S rRNA	16R-1492*#	TACGGCTACCTTGTTACGACTT	(Gomila, Ramirez et al. 2007)
23S rRNA	23S1R*	CCCAAAGCCTATATATTCAGC	(Matsumoto, Chimara et al. 2012)
rpoB	MYCOF1*	TCCGATGAGGTGCTGGCAGA	(Macheras, Roux et al. 2011)
rpoB	MYCOR2*	ACTTGATGGTCAACAGCTCC	(Macheras, Roux et al. 2011)
hsp65	Tb11	ACCAACGATGGTGTGTCCAT	(Telenti, Marchesi et al. 1993)
hsp65	Tb12	CTTGTCGAACCGCATACCCT	(Telenti, Marchesi et al. 1993)
hsp65	hsp667F*	GGCCAAGACAATTGCGTACG	(Selvaraju, Khan et al. 2005)
hsp65	hsp667R*	GGAGCTGACCAGCAGGATG	(Selvaraju, Khan et al. 2005)
ITS	Sp1*	ACCTCCTTTCTAAGGAGCACC	(Roth, Reischl et al. 2000)
ITS	Sp2	GATGCTCGCAACCACTATCCA	(Roth, Reischl et al. 2000)
ITS	23S1R*	CCCAAAGCCTATATATTCAGC	(Matsumoto, Chimara et al. 2012)

*Primers used for PCR amplification and sequencing of 16S rRNA, *hsp*65, *rpoB* and ITS
Primers were modified based on *M. abscessus* subsp. *abscessus* ATCC 19977 genome

Table S9.3. Species, strains and GenBank accession numbers of sequences used for phylogenetic analyses. Number of characters in each DNA sequence alignment shown in final row.

***Mycobacterium* species**	**Strain**	**16S rRNA**	***hsp65***	***rpoB***
M. abscessus subsp. *abscessus*	CIP 104536^{T}	AY457071	AY458075	AY147164
M. abscessus subsp. *boletti*	CIP 108541^{T}	AY859681	AY859675	AY859692
M. aubagnense	CIP 108543^{T}	AY859683	AY859677	AY859694
M. chelonae	CIP104535^{T}	AY457072	AY458074	AY147163
M. fortuitum	CIP 104534^{T}	AY457066	AY458072	AY147165
M. franklinii	DSM 45524^{T}	HQ153090	KM392059	HQ153091
M. immunogenum	CIP 106684^{T}	AY457080	AY458081	AY262739
M. mucogenicum	ATCC 49650^{T}	AY457074	AY458079	AY147170
M. phocaicum	CIP 108542^{T}	AY859682	AY859676	AY859693
M. salmoniphilum	ATCC 13758^{T}	DQ866768	DQ866777	DQ866790
M. saopaulense	JAN1	DQ866774	DQ866786	DQ866800
M. saopaulense	JAN2	DQ866775	DQ866787	DQ866801
M. saopaulense	EPM 10695	KM973036	KM973025	KM973028
M. saopaulense	EPM 10906^{T}	KM973037	KM973026	KM973029
M. saopaulense	IAL 3785	KM973038	KM973027	KM973030
Characters in alignment		1387	401	676

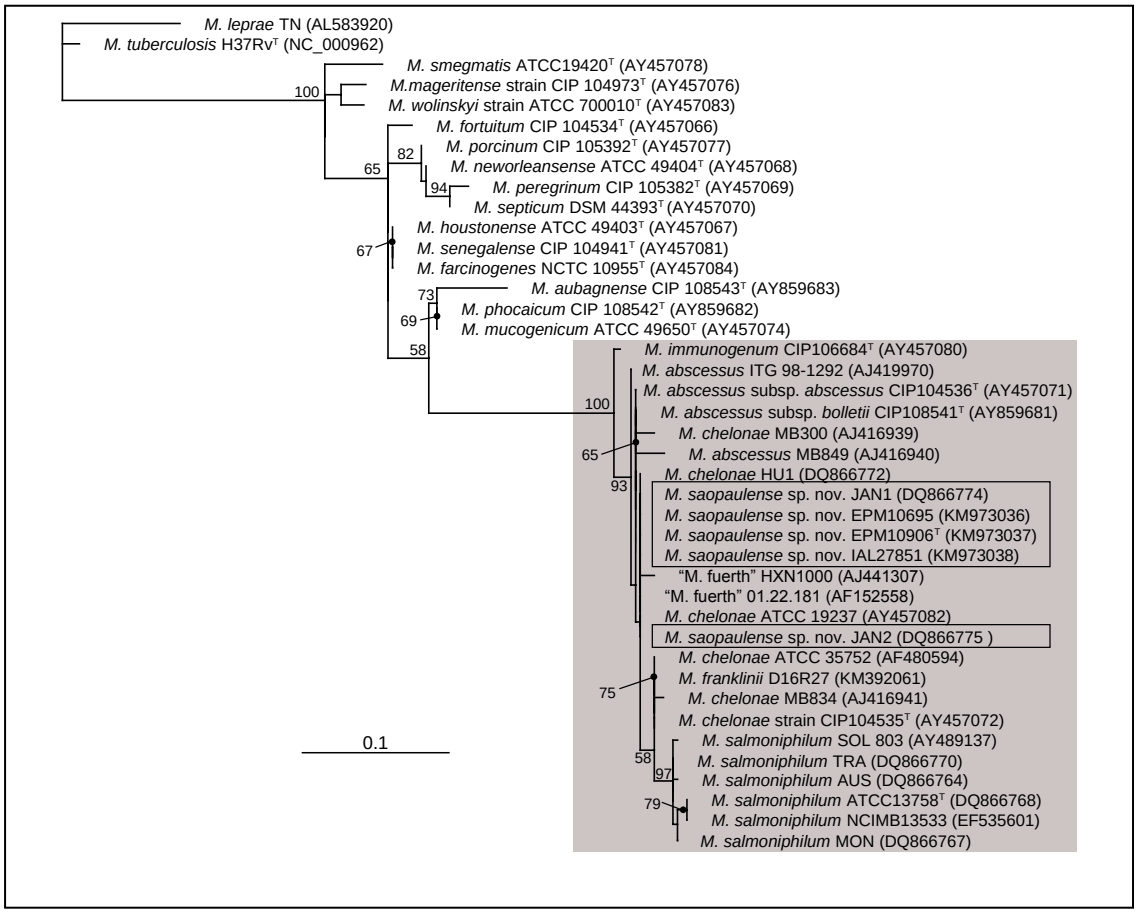


Figure S9.1. Estimate of phylogeny of *Mycobacterium* species based on small subunit (16S) ribosomal DNA sequences. Names and strains are shown, with Genbank accession numbers in parentheses. A superscript 'T' represents sequences from type species. Bootstrap values greater than 50% are shown at nodes. The *M. chelonae-M. abscessus* group has 100% bootstrap support and is shown in a grey box. Within the group, most relationships are unresolved using 16S, and species are intermixed with the exception of *M. immunogenum* and *M. salmoniphilum*.

10 Characterization of *Mycobacterium chelonae*-like Strains by Comparative Genomics

Christiane Lourenço Nogueira, Luiz Gonzaga Paula de Almeida, Maria Carmen Menendez, Maria Jesus Garcia, Luciano Antonio Digiampietri, Erica Chimara, Margo Cnockaert, Juan Carlos Palomino, Anandi Martin, Peter Vandamme, Sylvia Cardoso Leão. Genomic variability of *Mycobacterium chelonae* (*Submitted to Frontiers in Microbiology*).

Abstract

Isolates of the *Mycobacterium chelonae-M. abscessus* complex are subdivided into four clusters (CHI to CHIV) in the INNO-LiPA® *Mycobacterium* spp DNA strip assay. A considerable phenotypic variability was observed among isolates of the CHII cluster. In this study, we examined the diversity of 26 CHII cluster isolates by phenotypic analysis, drug susceptibility testing, whole genome sequencing and single-gene analysis. Pairwise genome comparisons were performed using several approaches, including average nucleotide identity (ANI) and genome-to-genome distance (GGD) among others. Based on ANI and GGD the isolates were identified as *M. chelonae* (14 isolates)*, M. franklinii* (2 isolates) and *M. salmoniphium* (1 isolate). The remaining 9 isolates were subdivided into three novel putative genomospecies. Phenotypic analyses including drug susceptibility testing, as well as whole genome comparison by TETRA and delta differences, were not helpful in separating the groups revealed by ANI and GGD. The analysis of standard four conserved genomic regions showed that *rpoB* alone and the concatenated sequences clearly distinguished the taxonomic groups delimited by whole genome analyses. In conclusion, the CHII INNO-LiPA® is not a homogeneous cluster; on the contrary, it is composed of closely related different species belonging to the *M. chelonae-M. abscessus* complex and also several unidentified isolates. The detection of these isolates, putatively novel species, indicates a wider inner variability than the presently known in this complex.

10.1 Introduction

The *Mycobacterium chelonae-M. abscessus* complex consists of closely related rapidly growing mycobacteria. Although ubiquitous environmental organisms, they can cause several opportunistic infections in humans, especially pulmonary and skin infections (Wallace, Swenson et al. 1983, Brown-Elliott and Wallace 2002, Whipps, Butler et al. 2007). This complex is the most commonly identified mycobacterial group causing diseases in humans after the *Mycobacterium tuberculosis* and *Mycobacterium avium* complexes (Sassi and Drancourt 2014). Nowadays, *M. abscessus* is one of the main infectious agents causing respiratory exacerbation in patients with cystic fibrosis (Bryant, Grogono et al. 2013).

Several changes in the classification of the members of the *M. chelonae-M. abscessus* complex have occurred over the years. Currently the species that are formally accepted include *M. chelonae*, *M. abscessus* (Kusunoki and Ezaki 1992) – with three subspecies*, M. abscessus* subsp. *abscessus, M. abscessus* subsp*. massiliense* and *M. abscessus* subsp. *bolletii* (Leao, Tortoli et al. 2009, Leao, Tortoli et al. 2011, Tortoli, Kohl et al. 2016), *M. immunogenum* (Wilson, Steingrube et al. 2001), *M. salmoniphilum* (Whipps, Butler et al. 2007), *M. franklinii* (Simmon, Brown-Elliott et al. 2011, Lourenco Nogueira, Simmon et al. 2015) and M. saopaulense (Nogueira, Whipps et al. 2015).

Despite technological advances, accurate species level identification of *M. chelonae- M. abscessus* complex bacteria represents a challenge for clinical laboratories. In general, these species have indistinguishable phenotypic and biochemical characteristics (Simmon, Brown-Elliott et al. 2011, Lourenco Nogueira, Simmon et al. 2015, Nogueira, Whipps et al. 2015). Moreover, partial 16S rDNA sequences are too similar, underestimating their diversity and not distinguishing all taxa (Adekambi, Colson et al. 2003, Simmon, Brown-Elliott et al. 2011). *M. chelonae- M. abscessus* complex members can be differentiated by the analysis of DNA polymorphisms in the *rpoB* and *hsp65* genes and in the 16S-23S rRNA internal transcribed spacer (ITS-1). However, Adekambi et al. (2003) demonstrated that *M. abscessus* isolates have >4.3% *rpoB* sequence divergence, which is a considerable intra species variability that adds another

challenge to the identification of *M. chelonae- M. abscessus* complex bacteria (Adekambi, Colson et al. 2003).

A considerable variability was also observed among *M. chelonae* isolates during the development of a DNA strip assay named INNO-LiPA® *Mycobacterium* spp (Innogenetics, Belgium). This reverse hybridization line probe assay was developed based on the high ITS-1 sequence heterogeneity of mycobacteria. DNA probes specific for the clinically important mycobacterial species were selected, including a set of 9 probes specific for the *M. chelonae-M. abscessus* complex that allowed the subdivision of isolates from this group into four clusters (CHI, CHII, CHIII and CHIV) according to their hybridization profiles (Portaels, Rigouts et al. 1998). The commercial version of this test used only 3 probes, MCH-1, MCH-2 and MCH-3. Isolates that showed hybridization with probes MCH-1 and MCH-3 were identified as cluster CHI. Cluster CHIII showed hybridization with probes MCH-1 and MCH-2, and clusters CHII and CHIV only with probe MCH-1. *M. abscessus* isolates and the type strain ATCC 19977^T were encompassed in the CHIII cluster (Portaels, Rigouts et al. 1998). Interestingly, a high variability in phenotypic characteristics of isolates belonging to CHII cluster was observed, suggesting the existence of different taxonomic entities within the group. For example, *M. chelonae* isolates cannot grow in the presence of 5% NaCl and can use citrate as the sole carbon source (Yakrus, Hernandez et al. 2001). However some CHII isolates showed conflicting results by these tests (Portaels, Rigouts et al. 1998).

To explore the variability observed during the development of INNO-LiPA® assay, a set of CHII cluster isolates was studied. Whole genome sequencing and pairwise genome comparisons were performed to better understand the diversity of the CHII cluster. The ability of DNA targets commonly used for identification of mycobacteria in discriminating the groups separated by genomic comparisons was also verified.

10.1 Materials and Methods

Isolates, reference strains and growth media

This study was carried out with 26 isolates belonging to INNO-LiPA® cluster CHII recovered from clinical and environmental specimens by Prof. Françoise Portaels (Institute of Tropical Medicine Prince Leopold, Antwerp, Belgium) and Prof. Roland Schulze-Röbbecke (University of Dusseldorf, Dusseldorf, Germany). *M. smegmatis* mc^2155, *M. tuberculosis* H37Rv and the type strains of *M. chelonae- M. abscessus* complex (*M. abscessus* subsp. *abscessus* ATCC 19977^T, *M. abscessus* subsp. *bolletii* CCUG 50184^T, *M. abscessus* subsp*. massiliense* CCUG 48898^T*, M. chelonae* ATCC 35752^T, *M. immunogenum* ATCC 700505^T, *M. salmoniphilum* ATCC 13758^T, *M. franklinii* DSM 45524^T and *M. saopaulense* CCUG 66554^T) were included for comparison (Table 10.1).

Cultures were grown aerobically at 28-30^oC on solid media including Löwenstein-Jensen (LJ) and Middlebrook 7H10 (Becton-Dickinson (BD), USA) supplemented with oleic acid, albumin, dextrose and catalase (OADC – BD) and in liquid media including Middlebrook 7H9 (BD), Mueller-Hinton and Lisogeny Broth with 1% Tween 80.

Table 10.1. Isolates and type strains of *M. chelonae-M. abscessus* complex included in this study.

Isolate	Isolation Source	Procedence	INNO-LiPA®
96-1705	Human foot biopsy	ITM, Belgium	CHII
96-1717	Human hand tissue	ITM, Belgium	CHII
96-1720	Human leg abscess	ITM, Belgium	CHII
96-1724	Human leg abscess	ITM, Belgium	CHII
96-1728	Lizard liver tissue	ITM, Belgium	CHII
D16R24	Water work	UD, Germany	CHII
D16Q13	Tap water	UD, Germany	CHII
D16Q14	Tap water	UD, Germany	CHII
D16Q15	Tap water	UD, Germany	CHII
D16Q16	Water work	UD, Germany	CHII
D16Q20	Water work	UD, Germany	CHII
D16Q24	Tap water	UD, Germany	CHII
D16R2	Tap water	UD, Germany	CHII
D16R3	Tap water	UD, Germany	CHII
D16R7	Tap water	UD, Germany	CHII
D16R9	Tap water	UD, Germany	CHII

D16R10	Surface water	UD, Germany	CHII
D16R12	Surface water	UD, Germany	CHII
D16R14	Tap water	UD, Germany	CHII
D16R18	Tap water	UD, Germany	CHII
D16R19	Surface water	UD, Germany	CHII
D16R20	Surface water	UD, Germany	CHII
D17A2	Water work	UD, Germany	CHII
D16Q19	Water work	UD, Germany	CHII
D16R27	Tap water	UD, Germany	CHII
96-892	-	ITM, Belgium	CHII
M. abscessus subsp. abscessus	-	ATCC 19977^T	CHIII
M. abscessus subsp. bolletii	-	CCUG 50184^T	ND
M. massiliense	-	*CCUG 48898^T	ND
M. chelonae	-	ATCC 35752^T	CHII/IV
M. immunogenum	-	ATCC 700505^T	ND
M. salmoniphilum	-	ATCC 13758^T	ND
M. franklinii	-	DSM 45524^T	ND
M. saopaulense	-	CCUG 66554^T	ND

(UD): *University of Dusseldorf*, Dusseldorf, Germany; (ITM): *Institute of Tropical Medicine Prince Leopold*, Antwerp, Belgium; (Unk): Unknown; (ND): not determined
* now classified as *M. abscessus* subsp. *bolletii*

Phenotypic analyses

Phenotypic analyses were performed as described in standard protocols for biochemical identification of mycobacteria (Tsukamura 1984, Kent and Kubica 1985, Leão, Martin et al. 2004). Analysis of pigment production, single-source carbon utilization (mannitol, inositol and citrate), growth at 26^{o}C and 37^{o}C and tolerance to 5% NaCl, 0.2% picric acid, para-nitrobenzoic acid (PNB) and nitrite were performed on 7H10-OADC and LJ. Nitrate reduction, Tween 80 hydrolysis and arylsulfatase production were also examined.

Susceptibility testing

Antimicrobial drug-susceptibility testing was performed using the microdilution method in cation-supplemented Mueller–Hinton broth, according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI 2011) for rapidly growing mycobacteria. The antimicrobials tested were amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxycycline, minocycline, moxifloxacin and tobramycin.

DNA extraction

Chromosomal DNA was extracted using QIAamp DNA mini kit (Qiagen, Germany) as previously described (Bryant, Grogono et al. 2013). DNA concentration was determined using a Qubit high-sensitivity (HS) assay kit (Life Technologies, USA).

Whole Genome Sequencing and assembly

High quality DNA of the 26 isolates and of *M. abscessus* subsp. *bolletii* CCUG 50184^T, *M. immunogenum* ATCC 700505^T, *M. salmoniphilum* ATCC 13758^T and *M. franklinii* DSM 45524^T were subjected to multiplexed paired end sequencing using the Illumina Miseq platform. The genome of *M. saopaulense* CCUG 66554^T was sequenced in a previous project from the laboratory of the Universidade Federal de São Paulo (accession number CP010271). The genomes of *M. abscessus* subsp. *abscessus* ATCC19977^T (accession number CU458896), *M. abscessus* subsp. *massiliense* CCUG48898^T (accession number NZ_AKVF01000001 to NZ_AKVF01000005) and *M. chelonae* ATCC 35752^T (accession number CP010946) were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). Sequencing errors in reads were corrected with the program Quake v0.3 (Kelley, Schatz et al. 2010) and reads trimmed with the program Trimmomatic v0.33 (Bolger, Lohse et al. 2014). The assembly was performed with Newbler program v3.0 (20140318_1550) - version with support for reads with Illumina's Casava accession number v1.8 format) using default parameters. Raw sequencing data was deposited on the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) under accession number SRP075879 and the assembled genomes were deposited as BioProject PRJNA323571.

Procedures of whole genome sequence comparison

Average Nucleotide Identity (ANI) and Tetranucleotide frequency correlation coefficients (TETRA) analysis

Average Nucleotide Identity by BLAST (ANIb) and by MUMmer (ANIm) and TETRA-nucleotide usage patterns were calculated using JSpecies v1.2.1. Cutoff values for species separation were ≥ 95% ANIb and ANIm and ≥ 0.99

TETRA (Kurtz, Phillippy et al. 2004, Teeling, Meyerdierks et al. 2004, Goris, Konstantinidis et al. 2007). A tree based on the obtained ANIb values was constructed using MEGA7 software (Saitou and Nei 1987, Kumar, Stecher et al. 2016) using the genomes of *M. smegmatis* mc^2155 (accession number NC_018289) and *M. tuberculosis* H37Rv (accession number NC_018143) as outgroups.

Genome-to-Genome Distance (GGD) calculations

GGD was calculated using the Genome-to-Genome Distance Calculator (GGDC at http://ggdc.dsmz.de). The distance values between the genomes were determined and the digital DNA-DNA hybridization (dDDH) was calculated from these distances. Cutoff values for species differentiation were ≤ 0.0258 distance value and ≥ 70% dDDH (Meier-Kolthoff, Auch et al. 2013). A tree based on GGD values was constructed using MEGA7 software (Saitou and Nei 1987, Kumar, Stecher et al. 2016) using the genomes of *M. smegmatis* mc^2155 (accession number NC_018289) and *M. tuberculosis* H37Rv (accession number NC_018143) as outgroups.

Genomic Signature (delta values)

The relative abundance of di-, tri- and tetra-nucleotides distributed along the genomes was calculated using the program available at http://www.cmbl.uga.edu/software.htm. The delta value obtained by comparing a genome with itself is considered the threshold for species separation for that particular genome (Karlin, Mrazek et al. 1997).

Comparison of isolates by single-gene sequencing

Taxonomically informative partial sequences of 16S rDNA, *rpoB*, *hsp65* and 16S-23S ITS-1 fragments were PCR amplified and sequenced using primers listed in Table S10.1. PCR products were purified using QIAquick PCR purification Kit (Qiagen, Germany). Dideoxy sequencing was performed using BigDye® 19 Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and run in ABI PRISM 3100 DNA Analyzer (Applied Biosystems).

Individual and concatenated phylogenetic trees based on the partial sequences of the previous genomic regions were constructed using PhyML

(http://www.atgc-montpellier.fr/phyml/) (Guindon and Gascuel 2003), using as input the multiple alignment of these sequences produced by MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/help/) with default parameters (penalty for gap opening = 400, gap extension = 0). Confidence bootstrap values were calculated with 100 replicates. The corresponding sequences of the *M. chelonae- M. abscessus* complex type strains and outgroups (*M. tuberculosis* H37Rv and *M. smegmatis* mc2155) were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) (Table S10.2).

GenBank/EMBL/DDBJ accession numbers

The 16S rDNA, *rpoB*, *hsp65* and 16S-23S ITS-1 partial sequences of the isolates under study were deposited in the GenBank/EMBL/DDBJ under accession numbers: KT779787 to KT779815 (16S rDNA), KT779816 to KT779844 (*hsp65*), KT779874 to KT779902 (*rpoB*) and KT779845 to KT779873 (16S-23S ITS-1).

The genomes were deposited in the GenBank/EMBL/DDBJ under accession numbers: MAEQ00000000 (*M. chelonae* 96-1705), MAER00000000 (*M. chelonae* 96-1717), MAES00000000 (*M. chelonae* 96-1720), MAET00000000 (*M. chelonae* 96-1724), MAEU00000000 (*M. chelonae* 96-1728), MAEV00000000 (*M*. sp. D16R24), MAEP00000000 (*M. franklinii* D16R27), MAEW00000000 (*M*. sp. D16Q13), MAEX00000000(*M*. sp. D16Q14), MAEY00000000 (*M*. sp. D16Q16), MAFS00000000 (*M. franklinii* D16Q19), MAEZ00000000 (*M*. sp. D16Q20), MAFA00000000 (*M. chelonae* D16Q24), MAFB00000000 (*M*. sp. D17A2), MAFC00000000 (*M*. sp. D16R12), MAFD00000000 (*M*. sp. D16R18), MAFE00000000 (*M. salmoniphilum* D16Q15), MAFF00000000 (*M. chelonae* D16R2), MAFG00000000 (*M. chelonae* D16R3), MAFH00000000 (*M. chelonae* D16R7), MAFI00000000 (*M. chelonae* D16R9), MAFJ00000000 (*M. chelonae* D16R10), MAFK00000000 (*M. chelonae* D16R14), MAFL00000000 (*M. chelonae* D16R19), MAFM00000000 (*M. chelonae* D16R20), MAFN00000000 (*M*. sp. 96-892), MAFO00000000 (*M. abscessus* subsp. *bolletii* BD), MAFP00000000 (*M. immunogenum* MC 779), MAFQ00000000 (*M. franklinii* CV002), MAFR00000000 (*M. salmoniphilum* SC).

10. 2 Results

Phenotypic analyses and Susceptibility testing

All CHII isolates grew in the presence of PNB. They did not reduce nitrate or hydrolyze Tween 80, but they exhibited arylsulfatase activity within 3 days. All CHII isolates grew in the presence of 5% NaCl at 30^{o}C, but 9/26 isolates did not tolerate it at 37^{o}C. It is expected that all rapidly growing mycobacteria tolerate 0.2% nitrite and 0.2% picric acid (Tsukamura 1984). Among the isolates studied in the present work, 8/26 did not grow in the presence of nitrite. However, all isolates grew in the presence of picric acid and generated visible colonies on solid culture media within 7 days. Additionally, 5/26 isolates were not capable of growing in the presence of citrate as single carbon source (Table S10.3).

All isolates tested were susceptible to clarithromycin and resistant to cefoxitin, except for *M. franklinii* DSM 45524^{T}, which was susceptible to cefoxitin (MIC=16 μg/mL). Variable results were obtained with the other tested antimicrobials (Table S10.4).

Whole Genome Sequencing and assembly

The number of assembled bases ranged from 4,768,278 to 5,548,818, with an average G+C content of 63.94%. The number of generated scaffolds ranged from 15 to 82 with an N50 size from 110,426 bp to 682,599 bp (Table S10.5).

Average Nucleotide Identity (ANI)

Pairwise ANIb and ANIm values of the *M. chelonae- M. abscessus* complex type strains were all below 95%, except between *M. abscessus* subsp. *abscessus* ATCC 19977^{T}, *M. abscessus* subsp. *bolletii* CCUG 50184^{T} and *M. abscessus* subsp*. massiliense* CCUG 48898^{T}. These results confirmed that the type strains represent distinct species within the *M. chelonae- M. abscessus* complex and that the strains ATCC 19977^{T}, CCUG 50184^{T} and CCUG 48898^{T} are appropriately classified into a single species, *M. abscessus* (Table 10.2 and Table S10.6).

Table 10.2 Taxonomic groups based on results of ANI, GGD, dDDH, TETRA and delta differences. (A) results between isolates inside each group and (B) results between each group of isolates and the *M. chelonae-M. abscessus* type strains.

Isolates	ANI		GGD		dDDH		TETRA		delta differences	
	A	B	A	B	A	B	A	B	A	B
96-1705 96-1717 96-1720 96-1724 96-1728 D16Q24 D16R2 D16R3 D16R7 D16R9 D16R10 D16R14 D16R19 D16R20	>95%	**>95% ATCC 35752^T**	<0.0258	>0.0258	>70%	<70%	>0.99	>0.99	21-27	21-27
D16Q15	-	**>95% ATCC 13758^T**	-	>0.0258	-	**>70% ATCC 13758^T**	>0.99	>0.99	21-27	21-27
D16Q19 D16R27	>95%	**>95% DSM 45524^T**	<0.0258	>0.0258	>70%	**>70% DSM 45524^T**	>0.99	>0.99	21-27	21-27
D16Q14 D16Q20 D16R24 D17A2	>95%	<95%	<0.0258	>0.0258	>70%	<70%	>0.99	>0.99	21-27	21-27
96-892 D16Q16 D16Q13	>95%	<95%	<0.0258	>0.0258	>70%	<70%	>0.99	>0.99	21-27	21-27
D16R12 D16R18	>95%	<95%	>0.0258	>0.0258	<70%	<70%	>0.99	>0.99	21-27	21-27

Based on ANIb and ANIm values, the CHII isolates could be separated into different taxonomic groups. The pairwise genome alignment of 14 isolates (96-1705, 96-1717, 96-1720, 96-1724, 96-1728, D16Q24, D16R2, D16R3, D16R7, D16R9, D16R10, D16R14, D16R19, D16R20) yielded >95% ANIb and ANIm values, showing that they belong to the same species. The ANI values between these isolates and the type strains yielded values slightly higher than 95% with ATCC 35752^T, showing that they could be classified into the species *M. chelonae* according to their ANI. Isolate D16Q15 yielded ANIb and ANIm values above 95% with ATCC 13758^T, showing that it belongs to the species *M. salmoniphilum* by this approach; and isolates D16Q19 and D16R27 yielded ANIb and ANIm values above 95% with DSM 45524^T, indicating that they belong to the species *M. franklinii,* thus confirming previously reported data (Lourenco Nogueira et al., 2015). The remaining nine isolates yielded ANI values below 95% with all type strains indicating a clear separation from the *M.*

chelonae- M. abscessus complex at the species level. These isolates could be grouped in three genomospecies using their ANI values: D16Q14, D16Q20, D16R24 and D17A2 (Genomospecies A), 96-892, D16Q13 and D16Q16 (Genomospecies B); and D16R12 and D16R18 (Genomospecies C). Pairwise ANI values of isolates within each genomospecies were above 95% (Table 10.2 and Table S10.6). The complete ANIb data distribution was represented in a tree (Figure 10.1). The tree obtained using ANIm data showed the same distribution (data not shown).

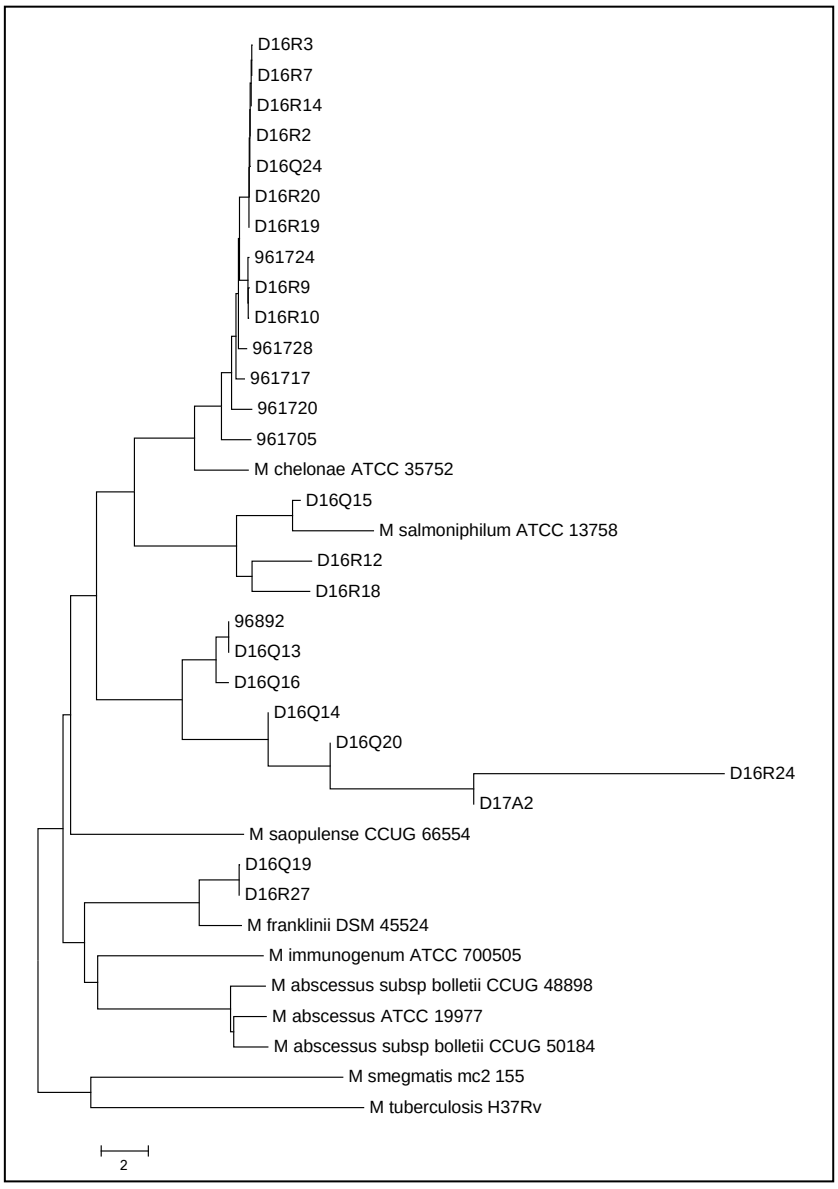


Figure 10.1 The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The tree based on ANIb analysis is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary analyses were carried out in MEGA7 (Kumar, Stecher et al. 2016).

Tetranucleotide frequency correlation coefficients (TETRA) analysis

The pairwise TETRA coefficients were all above the 0.99 threshold, even between the different type strains (Table 10.2). Isolates with pairwise ANI values above 95% showed TETRA values above 0.999 and pairwise ANI values below 95% corresponded to TETRA values between 0.99 and 0.999. ANI values below 95% and TETRA values above 0.999 were obtained in pairwise comparisons of D16Q16 and D16Q20, D16R18 and D16Q15, D16R18 and *M. salmoniphilum* ATCC 13758^T (Table S10.7). These results showed a low discriminative power of TETRA analysis applied to the CHII group.

Genome-to-Genome Distance (GGD) calculations

The genome distance displayed by the genomes under study confirmed the grouping obtained with ANI, however some partially discordant data were observed (Table 10.2 and Figure 10.2, Table S10.8). The 14 isolates found to belong to *M. chelonae* by ANI, showed data suggesting that they could belong to a different but very closely related species (GGD around 0.045 and dDDH around 64%) (Table S10.8). A similar result was found when comparing genomospecies C genomes each other (isolates D16R12 and D16R18) (Table S10.8), while GGD and dDDH data confirmed data for genomospecies A and B (Table 10.2).

On the other hand, isolate D16Q15 vs. *M. salmoniphilum* and D16Q19-D16R27 vs. *M. franklinii* showed GGD values slightly higher than the accepted threshold (0.033-0.034, see Table S10.6); yet, dDDH percentage values higher than 70% were obtained for both pairs of strains (Table 10.2).

As expected, the genomes from reference type-strains showed GGD and dDDH values corresponding to those of different species (GGD between 0.1272-0.1627; dDDH <33%; Table S10.6). Again, genome distance analysis showed values slightly higher than the threshold (0.0266-0.0288) corresponding to dDDH >70%, when comparing genomes of *M. abscessus* subspecies to each other (Table S10.8).

Data obtained of the genome distance, represented in a tree (Figure 10.2), showed similar genome distribution to that derived from ANI data (Figure 10.1).

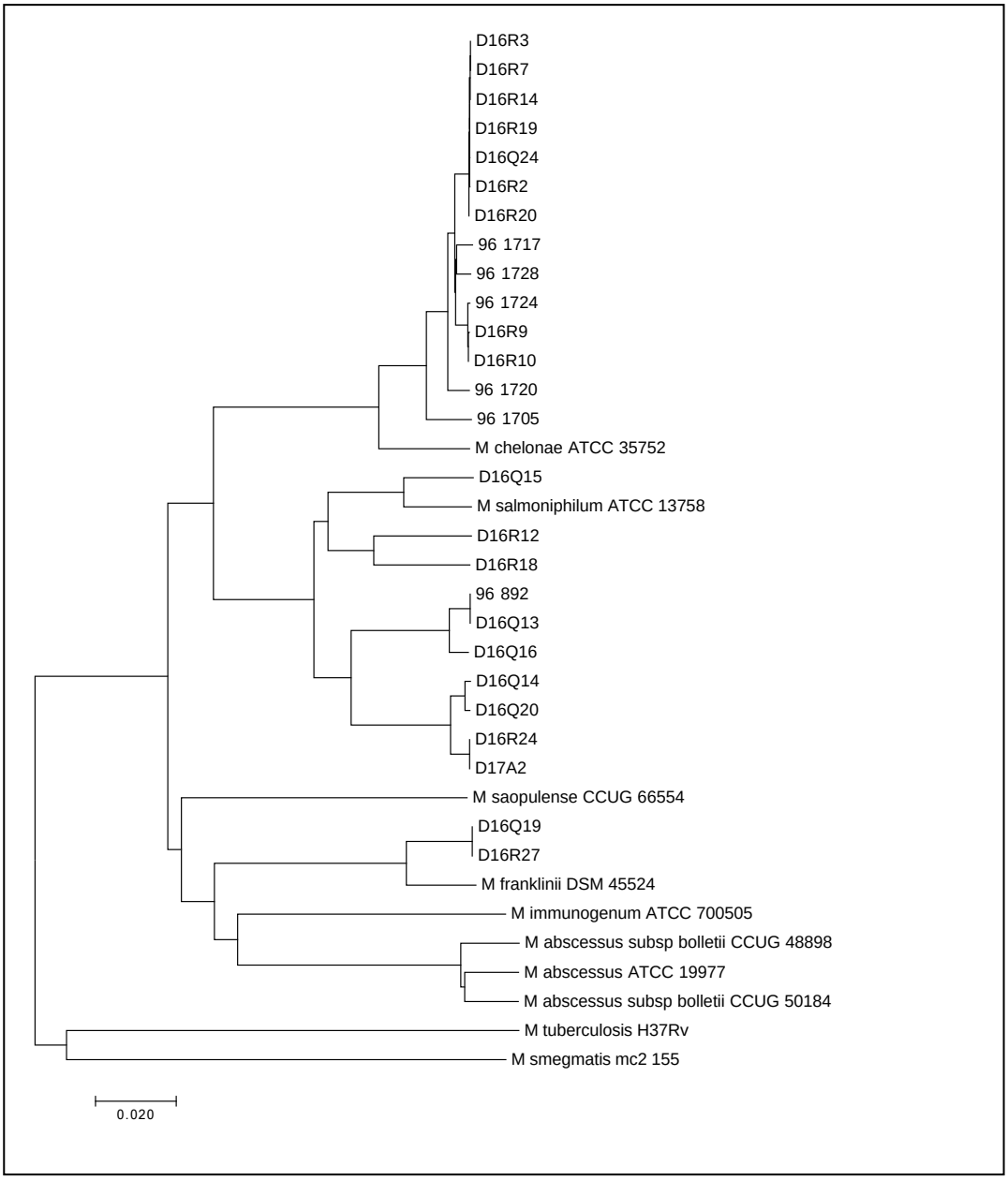


Figure 10.2 The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The tree based on GGD analysis is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary analyses were carried out in MEGA7 (Kumar, Stecher et al. 2016).

Genomic signature (delta values)

The obtained delta values fell within the range of the calculated cutoff values, between 21 and 27, indicating that all isolates and type strains are closely related. The type strains *M. abscessus* subsp. *abscessus* ATCC 19977^T, *M. abscessus* subsp. *bolletii* CCUG 50184^T and *M. abscessus* subsp. *massiliense* CCUG 48898^T showed the lowest delta values (22 to 24) and, as expected, were grouped as a single species. In accordance with the distribution found with ANI and GGD, the three *M. abscessus* subspecies and *M.*

immunogenum ATCC 700505^T appeared more separated from the other strains and isolates within the group (delta values of 24 to 28) (Table 10.2 and Table S10.9).

Single-gene analyses

Individual trees obtained with 16S rDNA, *rpoB*, *hsp65* and ITS-1 sequences grouped the CHII isolates among members of *M. chelonae-abscessus* complex (Figure 10.3 and Figure S10.1).

The individual trees obtained with 16S rDNA, *hsp65*, and ITS sequences showed some discordant groupings when compared to ANI and GGD trees (Figure S10.1). In the 16S rDNA tree all *M. chelonae* isolates clustered with *M. saopaulense* CCUG 66554^T. Moreover, it was not possible to discriminate *M. chelonae* ATCC 35752^T from *M. franklinii* isolates as well isolates of genomospecies A and genomospecies B. In the *hsp65* tree, isolates of genomospecies A clustered with *M. salmoniphilum* ATCC 13758^T and isolates D16Q19 and D16R27 did not cluster with *M. franklinii* DSM 45524^T. Moreover isolate D16Q15 did not cluster with *M. salmoniphilum* ATCC 13758^T. In the 16S-23S ITS tree, isolate D16Q15 clustered with isolates of genomospecies A and not with *M. salmoniphilum* ATCC 13758^T. Furthermore, isolates of genomospecies C were not grouped. In the *rpoB* and the concatenated trees all *M. chelonae* isolates clustered with *M. chelonae* ATCC 35752^T, D16R27 and D16Q19 with *M. franklinii* DSM 45524^T, and D16Q15 with *M. salmoniphilum* ATCC 13758^T. Moreover, genomospecies A, B and C formed clusters separated from all type strains (Figure 10.3). Therefore, *rpo*B and concatenated trees were in agreement with the isolates distribution obtained using ANI and GGD procedures.

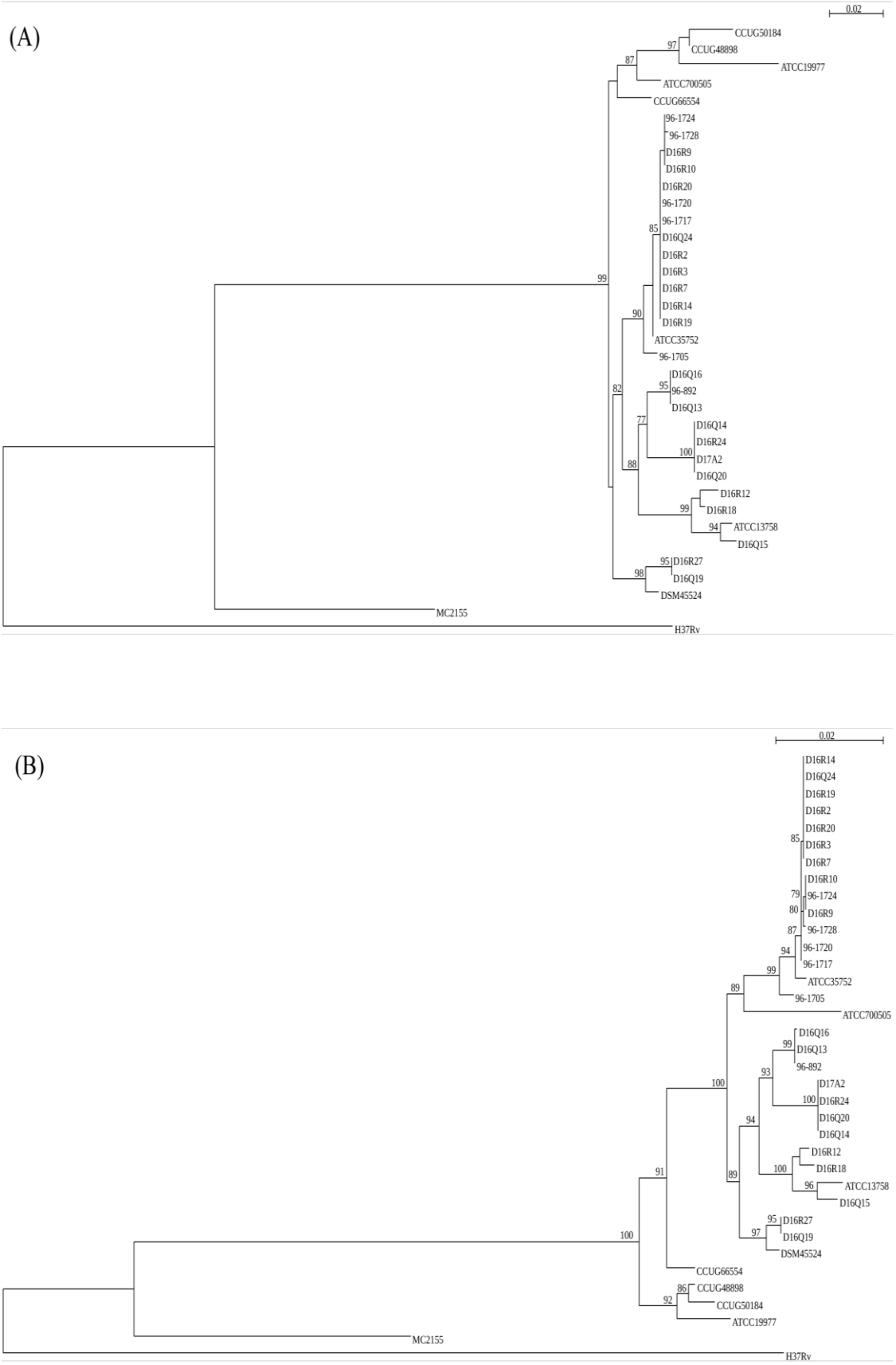


Figure 10.3 Trees based on the figure generated by SeaView (http://doua.prabi.fr/software/seaview). Bootstrap values >50% are shown at nodes. (**A**) *rpoB* (711 bp); (**B**) concatenated sequences of 16S rDNA (1384 bp), *hsp65* (401 bp), 16S-23S ITS fragment (214 bp) and *rpoB* (711 bp).

10.3 Discussion

The variability observed during the development of the INNO-LiPA® assay suggested the existence of different taxonomic groups among the CHII isolates. In the present study, we performed various whole genome sequence based analyses along with single gene sequencing and a biochemical characterization to characterize 26 INNO-LiPA® cluster CHII isolates and included type strains of the established species belonging to the *M. chelonae-M. abscessus* complex.

Similarly to previously published data (Lourenco Nogueira, Simmon et al. 2015, Nogueira, Whipps et al. 2015), phenotypic analyses and drug susceptibility tests were not helpful for distinguishing the taxonomic groups delimited by genomic analyses in this study.

Genomic analyses confirmed that the three subspecies within *M. abscessus* indeed represent a single species. *M. abscessus* subsp. *abscessus* ATCC 19977^T, *M. abscessus* subsp. *bolletii* CCUG 50184^T and *M. abscessus* subsp*. massiliense* CCUG 48898^T showed a GGD value higher than the proposed cutoff (0.0266 to 0.0288 distance values); this result is in agreement with the recent data found by Tortoli and co-workers (Tortoli, Kohl et al. 2016) when describing the subspecies within *M. abscessus*. However, the calculated dDDH percentages were higher than 70%, therefore within the value expected for a single species (Table S10.6).

Analysis of the remaining type strains through the determination of ANIb, ANIm, TETRA, delta, GGD-dDDH values revealed that species delineation threshold values that are commonly used cannot consistently be applied to these closely related *Mycobacterium* species. This observation was further endorsed through the analysis of some of the cluster CHII isolates where e.g. ANI analyses demonstrated that some strains represented a single species while GGD and dDDH suggested they represented closely related yet distinct species (see below). In addition, the threshold level of 0.99 to discriminate species by means of their genomic TETRA values proved inadequate as the TETRA value of every pair of strains examined in the present study was consistently above 0.99, even in the case that other approaches, such as ANI, indicated that were different species. Similarly, delta values proved to be not discriminatory either.

Fourteen isolates showed >95% ANI values with *M. chelonae* ATCC 35752^T yet GGD values (0.0449 to 0.0458) and dDDH (<70%, i.e. 63.50 to 64.00) suggested they represented a distinct species, closely related to *M. chelonae*.

In a similar manner, ANI and dDDH values assigned the isolate D16Q15 to *M. salmoniphilum* and the isolates D16Q19, D16R27 to *M. franklinii* while GGD values suggested they represented distinct species, closely related to *M. salmoniphilum* ATCC 13758^T (0.0340 distance value) and *M. franklinii* DSM 45524^T (0.0336 distance values), respectively.

Finally, the genomic data also showed that the remaining nine isolates represent at least three novel species closely related to *M. salmoniphilum*. The genomic parameters for genomospecies A and B are consistent. For genomospecies C however, ANI values demonstrate that the isolates D16R12 and D16R18 represent a single species while GGD and dDDH data suggest they represent two species (0.0480 and 62.2%, respectively). When Whipps *et al*. proposed to revive the name *M. salmoniphilum* in 2007, a high variability among *M. salmoniphilum* isolates was observed (Whipps, Butler et al. 2007). Moreover, 16S rDNA and *hsp65* sequences of isolates of genomospecies A, B and C showed a high similarity with the respective sequences of isolates recovered from fish, especially salmonids, in different geographic regions - Japan, Russia, Norway, Scotland, USA and Chile - and from tap water in the Netherlands (Whipps, Butler et al. 2007, van Ingen, Blaak et al. 2010, Righetti, Favaro et al. 2014). Together, these findings indicate that the *M. salmoniphilum* lineage comprises a broad group of closely related species that could represent a species complex in its own right.

Whole genome sequencing is still not routinely available for diagnostic purposes, making the analysis of few genes or informative genomic regions the standard procedure to identify difficult mycobacteria. Individual and concatenated phylogenetic trees of taxonomically informative sequences were constructed to evaluate if they could accurately discriminate the species/groups established by ANI and GGD and be useful for the identification of these taxa in routine laboratories. Only the *rpoB* and the concatenated phylogenetic tree clearly showed the same taxonomic groups discriminated by ANI and GGD analyses (Figure 10.3), therefore, comparison of these sequence could

accurately be used in the identification of members of the *M. chelonae- M. abscessus* complex until WGS could enter into the laboratory diagnostic routine.

Our results showed that the current threshold values applied in the procedures used for WGS species delineation could probably not be of general application to all the bacterial taxa, as was the case of the *M. chelonae- M. abscessus* complex, probably due to the different evolutionary routes followed by different bacteria, revealed after a whole genome comparison.

Acknowledgments

We acknowledge Prof. Roland Schulze-Röbbecke (University of Dusseldorf, Dusseldorf, Germany) for providing isolates for this study. This work has been partially supported by International Cooperation UAM-Banco Santander and Latin America (CEAL-UAM).

Funding

This study received financial support from Fundação de Amparo à Pesquisa do Estado de São Paulo (www.fapesp.br) (FAPESP) (grant 2011/18326-4). CN received a fellowship from FAPESP (2012/13763-0).

Supplementary material

Figure S10.1 Trees based on the figure generated by SeaView. Bootstrap values >50% are shown at nodes. (A) 16S rDNA (1384 bp), (B) *hsp65* (401 bp), (C) 16S-23S ITS fragment (214 bp).

Table S10.1 Primers used for PCR amplification and partial sequencing of 16S rRNA, *hsp65*, *rpoB* and ITS fragments.

Gene	Primers	Sequence 5´-3´	References
16S rRNA	16S-27F*	AGAGTTTGATCCTGGCTCAG	(Harmsen, Dostal et al. 2003)
16S rRNA	800F	ATTAGATACCCTGGTAG	(Adekambi and Drancourt 2004b)
16S rRNA	16S-907R*	CCGTCAATTCCTTTGAGTTT	(Harmsen, Dostal et al. 2003)
16S rRNA	16R-1492*	TACGGCTACCTTGTTACGACTT	(Gomila, Ramirez et al. 2007)
rpoB	MycoF	GGCAAGGTCACCCCGAAGGG	(Adekambi, Colson et al. 2003)
rpoB	MycoR	AGCGGCTGCTGGGTGATCATC	(Adekambi, Colson et al. 2003)
hsp65	hsp667F	GGCCAAGACAATTGCGTACG	(Selvaraju, Khan et al. 2005)
hsp65	hsp667R	GGAGCTGACCAGCAGGATG	(Selvaraju, Khan et al. 2005)
ITS	Sp1	ACCTCCTTTCTAAGGAGCACC	(Roth, Reischl et al. 2000)
ITS	23S1R	CCCAAAGCCTATATATTCAGC	(Matsumoto, Chimara et al. 2012)

*Primers were modified based on the *M. abscessus* subsp. *abscessus* ATCC 19977^{T} genome

Table S10.2 *Mycobacterium* species, strains and GenBank accession numbers used for construction of the phylogenetic trees

Gene	*Mycobacterium* species	Strain	Accession number
16S rRNA	*M. abscessus* subsp. *abscessus*	CIP 104536^T	AY457071
	M. abscessus subsp. *bolletii*	CIP 108541^T	AY859681
	M. massiliense*	CCUG 48898^T	NR_043002
	M. chelonae	CIP 104535^T	AY457072
	M. immunogenum	DSM 45595^T	HE654001
	M. salmoniphilum	ATCC 13758^T	NR_043989
	M. franklinii	DSM 45524^T	HQ153090
	M. saopaulense	CCUG 66554^T	KM973037
	M. tuberculosis	H37Rv	NC_000962_3
	M. smegmatis	MC2 155^T	NC_008596
hsp65	*M. abscessus* subsp. *abscessus*	CIP 104536^T	AY458075
	M. abscessus subsp. *bolletii*	CIP 108541^T	AY859675
	M massiliense*	CCUG 48898^T	AY596465
	M. chelonae	CIP 104535^T	AY458074
	M. immunogenum	CIP 106684^T	AY458081
	M. salmoniphilum	ATCC 13758^T	DQ866777
	M. franklinii	DSM 45524^T	KM392059
	M. saopaulense	CCUG 66554^T	KM973026
	M. tuberculosis	H37Rv	NC_000962_3
	M. smegmatis	MC2 155^T	NC_008596
rpoB	*M. abscessus* subsp. *abscessus*	CIP 104536^T	AY147164
	M. abscessus subsp. *bolletii*	CIP 108541^T	AY859692
	M. massiliense	CCUG 48898^T	AY593981.2
	M. chelonae	CIP 104535^T	AY147163
	M. immunogenum	CIP 106684^T	AY262739
	M. salmoniphilum	ATCC 13758^T	KM392058
	M. franklinii	DSM 45524^T	KM392056
	M. saopaulense	CCUG 66554^T	KM973029
	M. tuberculosis	H37Rv	NC_000962_3
	M. smegmatis	MC2 155^T	NC_008596
ITS	*M. abscessus* subsp. *abscessus*	CIP 104536^T	AY593976
	M. abscessus subsp. *bolletii*	CCUG 50184^T	GU143888.2
	M. massiliense*	CCUG 48898^T	AY593978.1
	M. chelonae	ATCC 35752^T	AY498739
	M. immunogenum	CIP 106684^T	AY593977
	M. salmoniphilum	ATCC 13758^T	DQ866768
	M. franklinii	DSM 45524^T	HQ153093
	M. saopaulense	CCUG 66554^T	KM973034
	M. tuberculosis	ATCC 27294^T	L15623
	M. smegmatis	MC2 155^T	NC_008596

Table S10.3. Comparison of phenotypic and biochemical characteristics of the isolates and type strains included in this study.

Isolate	Pigment production	Growth in the presence of					Single-source carbon utilization			Nitrate reduction	Tween 80 hydrolysis	Arylsulfatase production
		Picric acid	PNB*	Nitrite	5%NaCl 30°C	5%NaCl 37°C	mannitol	inositol	citrate			
96-1705	nc	+	+	+	+	+	-	-	-	-	-	+
96-1717	nc	+	+	+	+	+	+	+	+	-	-	+
96-1720	nc	+	+	+	+	+	-	-	+	-	-	+
96-1724	nc	+	+	+	+	+	+	+	+	-	-	+
96-1728	nc	+	+	+	+	+	-	-	+	-	-	+
D16Q24	nc	+	+	+	+	+	-	-	+	-	-	+
D16R2	nc	+	+	+	+	+	+	+	+	-	-	+
D16R3	nc	+	+	+	+	-	-	-	-	-	-	+
D16R7	nc	+	+	+	+	+	-	+	+	-	-	+
D16R9	nc	+	+	-	+	+	-	-	+	-	-	+
D16R10	nc	+	+	-	+	+	-	-	+	-	-	+
D16R14	nc	+	+	+	+	+	-	-	+	-	-	+
D16R19	nc	+	+	+	+	-	-	-	+	-	-	+
D16R20	nc	+	+	+	+	+	-	-	+	-	-	+
D16Q15	nc	+	+	-	+	-	-	-	-	-	-	+
D16R12	nc	+	+	-	+	-	-	-	+	-	-	+
D16R18	nc	+	+	-	+	-	-	-	+	-	-	+
96-892	nc	+	+	+	+	+	-	-	+	-	-	+
D16Q13	nc	+	+	+	+	-	-	-	+	-	-	+
D16Q16	nc	+	+	-	+	-	-	-	+	-	-	+
D16Q14	nc	+	+	+	+	-	-	-	+	-	-	+
D16Q20	nc	+	+	-	+	-	-	-	+	-	-	+
D16R24	nc	+	+	-	+	+	-	-	-	-	-	+
D17A2	nc	+	+	+	+	+	-	-	-	-	-	+
D16Q19	nc	+	+	+	+	+	-	-	+	-	-	+
D16R27	nc	+	+	+	+	+	-	-	+	-	-	+
ATCC 19977^T	nc	+	+	+	+	+	-	-	-	-	-	+
CCUG 50184^T	nc	+	+	+	+	+	-	-	-	-	-	+
CCUG 48898^T	nc	+	+	+	+	+	-	-	-	-	-	+
ATCC 35752^T	nc	+	+	+	+	+	-	-	+	-	-	+
ATCC 700505^T	nc	+	+	+	+	+	+	-	+	-	-	+
ATCC 13758^T	nc	+	+	+	+	-	-	-	-	-	-	+
DSM 45524^T	nc	+	+	+	+	+	-	-	-	-	-	+
CCUG 66554T	nc	+	+	+	+	+	-	-	+	-	-	+

*PNB, *p*-aminobenzoic acid; **nc, nonchromogenic

Table S10.4. Antibiotic susceptibility profile of the isolates and the type strains included in this study.

Isolate	Amikacin	Cefoxitin	Ciprofloxacin	Clarithromycin	Doxycycline	Minocycline	Moxifloxacin	Tobramycin
96-1705	32*	**>512**	2*	≤0.5	**>32**	**16**	2*	2
96-1717	8	**>512**	1	≤0.5	**>32**	**16**	2*	2
96-1720	32*	**>512**	2*	≤0.5	**>32**	**32**	2*	4*
96-1724	8	**>512**	1	≤0.5	**>32**	**16**	0.5	1
96-1728	32*	**>512**	2*	≤0.5	**>32**	2*	2*	4*
D16Q24	32*	**>512**	2*	≤0.5	**>32**	**8**	2*	4*
D16R2	**64**	**>512**	**4**	≤0.5	**>32**	**32**	**4**	**8**
D16R3	32*	**>512**	0.5	≤0.5	**>32**	**8**	<0.25	4*
D16R7	32*	**>512**	**4**	≤0.5	**>32**	**16**	**4**	4*
D16R9	32*	**>512**	2*	≤0.5	**>32**	**16**	2*	4*
D16R10	16	**>512**	**4**	≤0.5	**>32**	**16**	1	2
D16R14	32*	**>512**	**8**	≤0.5	**>32**	**32**	**4**	4*
D16R19	**64**	**>512**	**4**	≤0.5	**>32**	**16**	**8**	4*
D16R20	8	**>512**	2*	≤0.5	**>32**	4*	1	1
D16Q15	**64**	**>512**	**4**	≤0.5	**>32**	**32**	**4**	**8**
D16R12	≤4	**>512**	≤0.25	≤0.5	≤0.25	1	0.5	**8**
D16R18	≤4	**>512**	0.5	≤0.5	≤0.25	0,5	1	**8**
96-892	32*	**>512**	0.5	≤0.5	**8**	0.5	0.5	**16**
D16Q13	32*	**>512**	1	≤0.5	**>32**	2*	2*	**32**
D16Q16	16	**512**	≤0.25	≤0.5	**>32**	**8**	≤0.25	4*
D16Q14	16	**>512**	2*	≤0.5	**>32**	**16**	2*	4*
D16Q20	8	**>512**	2*	≤0.5	**>32**	32	0.5	2
D16R24	16	**>512**	2*	≤0.5	**>32**	**16**	2*	4*
D17A2	-	-	-	-	-	-	-	-
D16Q19	8	64*	≤0.25	≤0.5	≤0.25	1	0.5	4*
D16R27	16	64*	≤0.25	≤0.5	≤0.25	≤0.25	1	**16**
ATCC 19977^{T}	8	64*	**4**	≤0.5	**>32**	**8**	**8**	**8**
CCUG 50184^{T}	16	32*	**8**	≤0.5	**>32**	**16**	**8**	**16**
CCUG 48898^{T}	8	**>512**	**4**	≤0.5	≤0.25	0.5	**4**	**8**
ATCC 35752^{T}	8	**512**	0.5	≤0.5	4*	2*	<0.25	2
ATCC 700505^{T}	≤4	**256**	1	≤0.5	**>32**	**8**	1	4*
ATCC 13758^{I}	16	**512**	1	≤0.5	**>32**	2*	2*	4*
DSM 45524^{T}	≤4	16	0.5	≤0.5	≤0.25	≤0.25	1	2
CCUG 66554^{T}	**128**	**>512**	2*	≤0.5	**>32**	**16**	**8**	**32**

Bold indicates resistance, * indicates intermediate resistance

Table S10.5. Illumina Miseq assembly details for the isolates and type strains of *M. chelonae-M. abscessus* complex

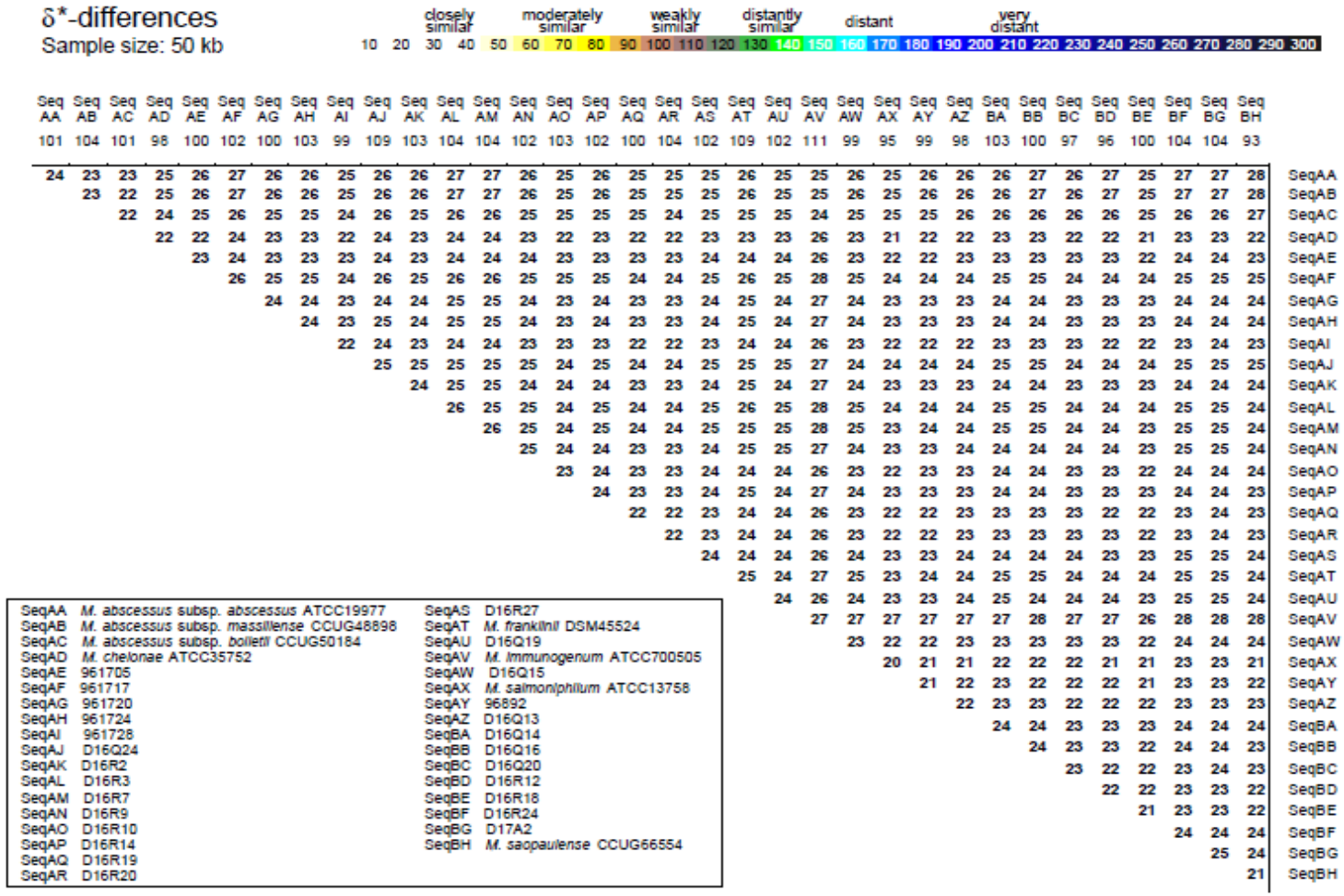
Isolates and type strains	Bases assembled	Number of scaffolds	N50 scaffolds size (bp)	Estimated %GC content
96-1705	4,998,263	29	270,676	63.98
96-1717	5,088,556	23	533,807	63.79
96-1720	4,989,632	27	281,017	63.89
96-1724	5,167,153	34	433,022	63.90
96-1728	4,940,199	22	569,132	64.07
D16R24	5,218,400	53	219,363	63.81
D16Q13	4,903,783	23	579,383	64.18
D16Q14	5,171,220	65	198,484	63.77
D16Q15	4,939,516	15	682,599	63.63
D16Q16	5,017,561	23	370,841	64.19
D16Q20	4,858,082	73	134,797	63.93
D16Q24	5,457,716	50	336,599	63.82
D16R2	5,153,513	23	350,269	63.85
D16R3	5,201,663	21	350,642	63.96
D16R7	5,212,684	20	500,734	63.89
D16R9	5,126,063	23	362,638	63.88
D16R10	5,133,965	48	187,245	63.90
D16R12	4,808,095	27	294,619	64.08
D16R14	5,123,766	21	575,060	63.91
D16R18	4,991,856	82	110,426	63.89
D16R19	4,989,084	24	306,045	63.95
D16R20	5,182,981	41	250,323	63.92
D17A2	5,201,188	56	219,356	63.76
D16Q19	5,112,070	38	477,240	63.93
D16R27	5,120,456	19	588,891	64.00
96-892	4,931,770	37	458,987	64.13
M. abscessus subsp. *boletii* CCUG 50184^T	5,044,397	21	443,298	63.90
M. immunogenum ATCC 700505^T	5,548,818	19	406,873	64.04
M. salmoniphilum ATCC 13758^T	4,768,278	25	250,904	64.15
M. franklinii DSM 45524^T	5,437,336	29	325,961	64.14

Table S10.6 ANIb and ANIm values obtained with the 26 isolates and the *M. chelonae-M. abscessus* type strains

Table S10.7 TETRA values obtained with the 26 isolates and the *M. chelonae-M. abscessus* type strains

Table S10.8 Genome to genome distance (GGD) and digital DNA-DNA hybridization (dDDH) values obtained with the 26 isolates and the *M. chelonae-M. abscessus* type strains

Table S10.9 Delta values obtained with the 26 isolates and the *M.chelonae-M. abscessus* complex type strains



PART IV: GENERAL DISCUSSION

11 General summary

The members of the *M. abscessus-M. chelonae* complex are medically among the most important NTM species. In the present study we examined the taxonomic position of a set of clinical and environmental isolates of the *M. chelonae-M. abscessus* complex previously included in the CHII cluster by the commercial DNA strip assay INNO-LiPA® and isolates that failed to match the recognized species patterns. Four isolates used for the development of INNO-LiPA® and the type strains of the species formally recognized as members of the *M. chelonae-M. abscessus* complex at the beginning of this study – *M. chelonae* (Bergey, Harrison et al. 1923), *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* (Leao, Tortoli et al. 2009, Leao, Tortoli et al. 2011), *M. immunogenum* (Wilson, Steingrube et al. 2001), and *M. salmoniphilum* (Whipps, Butler et al. 2007), plus *M. massiliense* (Adekambi, Reynaud-Gaubert et al. 2004a, Adekambi, Berger et al. 2006) and "*M. franklinii*" (Simmon, Brown-Elliott et al. 2011) were included for comparison.

In the first phase of this study (CHAPTER 7), the isolates were preliminarily characterized using GenoType® *Mycobacterium* CM and AS, PRA-*hps65* and PRA-ITS.

The GenoType® assay confirmed that all isolates included in this study belonged to the *M. chelonae-M. abscessus* complex.

Using PRA-*hsp65* and PRA-ITS, the representative strains of INNO-LiPA® CHI, CHII, CHIII and CHIV clusters showed different PRA-*hsp65* and PRA-ITS restriction profiles confirming that they belong to separate genotypes. These clusters were established during INNO-LiPA® development, since a set of *M. chelonae-M. abscessus* complex isolates showed different hybridization profiles with specific INNO-LiPA® DNA probes. When INNO-LiPA® was developed in the 1990s, the only formally recognized species of *M. chelonae-M. abscessus* complex were *M. abscessus* and *M. chelonae*. It was observed that INNO-LiPA® CHIII cluster encompassed *M. abscessus* and, accordingly, CHI, CHII and CHIV isolates were classified as *M. chelonae* (Portaels, Rigouts et al. 1998). In this work, isolate 8223, the representative strain of CHIII INNO-LiPA® cluster, was identified as *M. abscessus* by PRA-*hsp65* and PRA-ITS. The representative strain of CHIV (96-443) was

assigned to *M. chelonae*. However the identification of the representative strains of CHI (6410) and CHII (96-892) was not achieved, since discrepant results of PRA-*hsp65* and PRA-ITS were observed.

During INNO-LiPA® development high variability in phenotypic characteristics of CHII cluster isolates was observed, which is the reason why this group was evaluated in this thesis. In the first phase of this study high genotypic variability was observed among these CHII isolates and they were subdivided into six groups based on their PRA-*hsp65* and PRA-ITS restriction profiles. This high genotypic variability indicated that CHII isolates could indeed belong to distinct taxonomic groups, as suggested during the development of INNO-LiPA®. With the results of PRA-*hsp65* and PRA-ITS 16/28 CHII isolates were identified as *M. chelonae* and the taxonomic position of 12/28 isolates remained inconclusive, because they showed discordant results of PRA-*hsp65* and PRA-ITS (see Table 7.1). Isolates EPM 10906, EPM 10695, IAL 3785. JAN1 and JAN2 also remained without conclusive taxonomic classification after PRA-*hsp65* and PRA-ITS.

A polyphasic analysis was carried out in the second phase of this study in order to clarify the taxonomic position of the isolates with no conclusive species identification and to further explore the internal variability of CHII cluster isolates. Isolates were characterized using phenotypic analyses, drug susceptibility testing, DNA sequencing of the 16S rRNA, *hsp65* and *rpoB* genes and the 16S-23S ITS. Seven isolates showed results that confirmed that they belong to the *M. chelonae-M. abscessus* complex, but did not belong to any of the accepted species and therefore they likely represented clinically important new species. These isolates were then submitted to DDH experiments against the type strains of the *M. chelonae-M. abscessus* complex to confirm that they represented novel species of this complex. Most taxonomists agree that DDH is still the cornerstone in the delineation of bacterial species (Stackebrandt, Frederiksen et al. 2002, Goris, Konstantinidis et al. 2007, Ramasamy, Mishra et al. 2014).

Isolates D16Q19 and D16R27 (that belong to CHII INNO-LiPA® cluster) showed high 16S rRNA, *rpoB*, *hsp65* and 16S-23S ITS sequence identities with the respective sequences of "*M. franklinii*" DSM 45524^T. This species was proposed in 2011 by Simmon *et al*. when clinical isolates from patients with sinopulmonary disease were studied (Simmon, Brown-Elliott et al. 2011). However, this proposal

was effectively published in Emerging Infectious Diseases, and not in the IJSEM and the authors did not valide the effectively published name "*M. franklinii*". In order to confirm that this taxon is really a new species of *M. chelonae-M. abscessus* complex, isolate D16R27 was submitted to the DDH experiment against the type strains of the *M. chelonae-M. abscessus* complex. The average DDH value between D16R27 and "*M. franklinii"* DSM 45524^T was higher than the cutoff value of 70% confirming that both belong to the same species. Moreover, the average hybridization values of D16R27 with *M. abscessus* subsp. *abscessus* ATCC19977^T, *M. chelonae* ATCC 35752^T, *M. immunogenum* ATCC 700505^T and *M. salmoniphilum* ATCC 13758^T were all below 70%, confirming that isolate D16R27 represents a distinct species of the *M. chelonae-M. abscessus* complex. After phenotypic and genotypic characterization of these isolates, the name *M. franklinii* sp. nov. was formally published in IJSEM which validated its name (CHAPTER 8).

Isolates EPM 10906, EPM 10695, IAL 3785, JAN1, JAN2 also appeared to represent a clinically important new species. The two EPM isolates (EPM 10906 and EPM 10695) and IAL 3785 were obtained from clinical samples in different years and cities of Brazil, while isolates JAN1 and JAN2 were obtained from zebrafish in USA. They showed overlapping phenotypic and genotypic characteristics with members of *M. chelonae-M. abscessus* complex. Moreover, discordant results were observed in DNA sequencing results. The *hsp65*, *rpoB* and ITS sequences shared highest identity with the corresponding sequences of *M. abscessus* subsp. *abscessus* CIP 104536^T, *M. chelonae* CIP 104535^T and *M. franklinii* DSM 45524^T, respectively (ANNEX 1). DDH experiments confirmed that the five isolates belong to the same species since hybridization values above 70% were obtained. Then the isolate EPM 10906^T was selected to perform DDH with the type strains of the *M. chelonae-M. abscessus* complex. All values were below 70%, confirming that EPM 10906^T and the other four isolates belong to a distinct species of the *M. chelonae-M. abscessus* complex. The name *M. saopaulense* sp. nov. was proposed and validly published in IJSEM (CHAPTER 9).

The DDH technique needs considerable amounts of high quality DNA. Since mycobacteria have a complex cell wall structure that is impermeable and difficult to lyse, an effective DNA extraction is not easy. Most of the commonly used DNA isolation procedures result in poor quality and low yield of DNA. Consequently, to

achieve high amounts of high quality DNA more than 1 liter of liquid bacterial culture is required. Furthermore, to achieve the required DNA concentration it is necessary to repeat the DNA extraction protocol several times and pool the harvested DNA preparations. All these facts turn the whole process expensive, time-consuming and labor-intensive. With the introduction of NGS technology, the use of entire genome sequence analysis in taxonomic studies of prokaryotes has become an important tool to replace of DDH (Moore, Mihaylova et al. 2010).

Therefore in the third phase of this study (CHAPTER 10) the isolates of CHII INNO-LiPA® cluster were submitted to WGS in order to clarify their taxonomic position at species level. Although some isolates of the CHII cluster were already identified as *M. chelonae* in the first phase of this study, the taxonomic position of 10 isolates of the CHII INNO-LiPA® cluster (96-892, D16Q13, D16Q14, D16Q16, D16Q20, D16R24, D17A2, D16Q15, D16R12 and D16R18) still remained doubtful. All CHII isolates were submitted to WGS and pairwise genome comparisons in order to better understand the population structure and diversity of this cluster. Several pairwise genome comparisons, including ANI, TETRA analysis, genomic signature (delta values), GGD calculations and digital DNA-DNA hybridization, were performed to explore the relationships among the isolates and the species of the *M. chelonae-M. abscessus* complex. Unfortunately the DNA of isolates 96-1712 and D16R4 were contaminated and it was not possible to perform the genome assembly. These isolates were not included in the genome comparisons analyses.

Briefly, considering the results of the whole genome sequence comparisons, the 26 isolates from INNO-LiPA® cluster CHII could be identified as follows: 14 isolates as *M. chelonae*, two as *M. franklinii*, one as *M. salmoniphilum* and nine belonging to three novel genomospecies. These results confirmed that the CHII cluster encompasses different species of *M. chelonae-M. abscessus* complex.

However, discordant results were verified between ANI values and the other genome comparison analyses, suggesting that the cut-off values currently used for other bacteria could not be applied to closely relatated species of mycobacteria, such as the members of *M. chelonae- M.abscessus* complex.

Pairwise genome comparisons between the type strains of the *M. chelonae-M. abscessus* complex including the two new species described, *M. franklinii* DSM 45524^T and *M. saopaulense* CCUG 66554^T, were also performed in order to revisit

the current taxonomic classification of *M. chelonae-M. abscessus* complex. The ANI values between the type strains were all below 95%, except between *M. abscessus* subsp. *abscessus* ATCC 19977^T, *M. abscessus* subsp. *bolletii* CCUG 50184^T and *M. massiliense* CCUG 48898^T, which were above 95%. These results confirmed that the type strains represent distinct species of *M. chelonae-M. abscessus* complex and that strains ATCC 19977^T, CCUG 50184^T and CCUG 48898^T belong to the same species (*M. abscessus*).

Finally, at the end of the third phase of this study, the taxonomic position of the isolates was clarified. However, different approaches were needed to determine their taxonomic status. Phenotypical and some molecular tests used for mycobacteria identification were unable to correctly determine the assignment at species level of some isolates. Furthermore, some discrepancies among different methodologies were observed which could lead to erroneous identification at species level and consequently to implications in patient management. Discrepancies and some difficulties in species identification faced during this study will be discussed in the next sections of this chapter.

12 Discussions

12.1 Phenotypic identification

Biochemical and culture tests were poorly discriminative in this study. All isolates exhibited overlapping characteristics with the type strains and none of the phenotypic tests was able to discriminate the *M. chelonae-M. abscessus* complex species, especially those new species described in this study. Moreover, several tests needed to be repeated because interpretation of the results was doubtful. Previous studies commonly reported that phenotypic tests are time-consuming and lack discriminatory power (Butler and Guthertz 2001, Tortoli, Bartoloni et al. 2001, Cook, Turenne et al. 2003, Chimara, Ferrazoli et al. 2008, Simmon, Brown-Elliott et al. 2011). Interspecies homogeneity and intraspecies variability in phenotypic tests for identification of mycobacteria were previously observed by different authors (Butler and Guthertz 2001, Tortoli, Bartoloni et al. 2001, Cook, Turenne et al. 2003, Chimara, Ferrazoli et al. 2008, Simmon, Brown-Elliott et al. 2011).This explains in

part why they were replaced by molecular methods for mycobacteria species identification in clinical laboratories. However, it has been proposed that phenotypic characterization should be performed in taxonomic studies and for the descriptions of new species (Brown-Elliott and Wallace Jr. 2012b).

As observed during INNO-LiPA® development, isolates of the CHII cluster, which should be assigned to *M. chelonae*, showed a high variability in phenotypic test results and some phenotypic characteristics were compatible with an identification of *M. abscessus* (Françoise Portaels, personal communication). As reported by Kusunoki and Ezaki (1992), *M. abscessus* is distinguished from *M. chelonae* by tolerance to 5% NaCl and incapacity to use citrate as single-source-carbon. In the present study, all isolates that belong to *M. chelonae* tolerated 5% NaCl at 30°C and only two isolates didn't tolerate it at 37°C. Only two *M. chelonae* isolates did not grow in the presence of citrate.

According to standard protocols for phenotypic and biochemical identification of mycobacteria, culture tests should be performed on Löwenstein-Jensen medium and incubated at 37°C (Tsukamura 1984, Kent and Kubica 1985, Leão, Martin et al. 2004). However, some isolates of the *M. chelonae-M. abscessus* complex do not grow well on Löwenstein-Jensen. Moreover, their optimum growth occurs at 30°C (except for *M. abscessus* that grows better at 37°C). This is the reason why the tests performed in the present study were incubated at 30°C. It is possible that in this condition *M. chelonae* isolates tolerate 5% NaCl and can use citrate as single-source-carbon.

Inappropriate culture medium and incubation temperature could be responsible for misdiagnosis of mycobacterial infections. Routine laboratories should be alert to the possibility of an infection caused by *M. chelonae-M. abscessus* complex members, for example when the clinical specimen is from infections after traumatic injuries or surgeries. In these situations, cultures should be incubated also at 30°C.

12.2 Drug Susceptibility testing

The DST was performed by the microdilution method as recommended by CLSI; however, some problems were faced during its performance. Some tests had

to be repeated several times because the color of the positive control did not change to pink after one day of incubation with rezasurin; more often the color changed after two days of incubation. With some drugs, MIC determination was doubtful and it was necessary to perform the test several times until the interpretation was clear. The majority of isolates studied here formed large clumps when grown in liquid medium. Even when using a vortex to disperse the bacteria it was really hard to dissolve the clumps and therefore it was very difficult to adjust the inoculum suspension turbidity to 0.5 McFarland standard, explaining at least in part the problems faced during DST. MIC was not determined for isolate D17A2 that didn't grow in the test plates in five experiments.

DST has also been used in conjunction with molecular results to confirm the species identification since members of *M. chelonae-M. abscessus* complex have different antimicrobial susceptibility patterns (Brown-Elliott and Wallace 2002, Brown-Elliott and Wallace Jr. 2012b). *M. chelonae* generally has MIC ≥256 µg/mL for cefoxitin, whereas with *M. abscessus* MIC ranges from 32 to 64 µg/mL. Moreover, *M. abscessus* is generally resistant to tobramycin (MIC ≥ 8 µg/mL) while *M. chelonae* is susceptible (MIC ≤ 2 µg/mL) (Brown-Elliott and Wallace 2002). *M. immunogenum* is generally resistant to both cefoxitin and tobramycin (Wilson, Steingrube et al. 2001). These data are consistent with the results of the present study, except for isolate D16R2 (*M. chelonae*) that showed resistance to tobramycin and *M. immunogenum* ATCC 700505^T that showed intermediate resistance to tobramycin. *M. massiliense* was reported to be susceptible to doxycycline (MIC ≤ 1 µg/mL) (Adekambi, Reynaud-Gaubert et al. 2004a) and *M. abscessus* subsp. *bolletii* (CCUG 50184^T) to be resistant to most antimicrobial agents including clarithromycin (MIC ≥ 8 µg/mL) (Adekambi, Berger et al. 2006). In our study *M. massiliense* CCUG 48898^T was susceptible to doxycycline; however *M. abscessus* subsp. *bolletii* (CCUG 50184^T) was susceptible to clarithromycin. There are no consistent data about antimicrobial susceptibility patterns of *M. salmoniphilum* in the literature. In the present study the type strain ATCC 13758^T was resistant to cefoxitin and doxycycline, while D16Q15 was susceptible only to charithromycin.

The two new species described in this study showed particular susceptibility patterns. *M. franklinii* was more susceptible and *M. saopaulense* was more resistant

to antimicrobial drugs than the other isolates and members of the *M. chelonae-M. abscessus* complex.

From the clinical point of view, antibiotic therapy is often based on species identification alone, since some species present characteristic antimicrobial patterns. As described above variable results were found in comparison with data from the literature. Furthermore, variable results also occurred with isolates that belong to the same species. These findings highlight the necessity to perform DST in clinical isolates of the *M. chelonae-M. abscessus* complex to initiate adequate therapy.

12.3 GenoType® *Mycobacterium* CM and AS

As reported in several studies, GenoType® *Mycobacterium* is a rapid, easy-to-perform, and easy-to-interpret assay (Richter, Rusch-Gerdes et al. 2006, Russo, Tortoli et al. 2006, Neonakis, Gitti et al. 2008). Indeed, in our study the experiments did not need to be repeated and no technical difficulties or doubts in results interpretation occurred.

Using GenotyTpe® AS, all isolates and type strains studied showed the same hybridization profile (hybridization with probe 12) that corresponds to a species different from the mycobacteria species identifiable by this assay. In fact, the manufacturer does not recommend this assay to identify species of *M. chelonae-M. abscessus* complex. However these results show that there are no overlapping hybridization profiles between the species identifiable by GenotyTpe® AS and members of *M. chelonae-M. abscessus* complex, especially the species recently described, *M. salmoniphilum*, *M. franklinii* and *M. saopaulense*.

However GenoType® CM showed limitations regarding species identification of *M. chelonae-M. abscessus* complex isolates and failed to identify the new species. GenoType® CM correctly identified isolate 8223 and the type strains ATCC 19977^T, CCUG 50184^T and CCUG 48898^T as *M. abscessus* (hybridization with probes 5, 6 and 10). The other type strains and isolates were identified as *M. chelonae* (hybridization with probes 5 and 10). According to the manufacturer *M. immunogenum* can either show the hybridization profile of *M. chelonae* or that of *M. abscessus* and there is no information about overlapping profiles among the other

members of the *M. chelonae-M. abscessus* complex, which were validated after the introduction of this test.

Some previous studies reported overlapping profiles of GenoType® CM in other species of NTM. However this assay was considered suitable to identify clinically important mycobacteria (Richter, Rusch-Gerdes et al. 2006, Russo, Tortoli et al. 2006, Neonakis, Gitti et al. 2008). In the present study overlapping hybridization profiles were verified among *M. chelonae*, *M. immunogenum*, *M. salmoniphilum*, *M. franklinii* and *M. saopaulense*. Infections caused by *M. chelonae-M. abscessus* complex members have been increasingly reported (Griffith, Aksamit et al. 2007, Alcaide and Esteban 2010, Koh, Jeon et al. 2011, Nunes Lde, Baethgen et al. 2014) and, except for *M. salmoniphilum*, all these species have been recovered from humans (Meyers, Brown-Elliott et al. 2002, Sampaio, Junior et al. 2006, Del-Castillo, Palmero et al. 2009, Shedd, Edhegard et al. 2010, Mitchell, Isenstein et al. 2011, Simmon, Brown-Elliott et al. 2011, Biggs, Chudgar et al. 2012, Bhandari, Sriganesh et al. 2015, Chatzikokkinou, Luzzati et al. 2015, Edens, Liebich et al. 2015, Greninger, Langelier et al. 2015, Nogueira, Whipps et al. 2015, Shah, Relhan et al. 2016). This finding is an important issue for routine laboratories, especially in European countries, that use GenoType® for NTM species identification (van der Werf, Kodmon et al. 2014). We therefore conclude that our study demonstrated that GenoType® CM is suitable for identifying strains as belonging to *M. chelonae-M. abscessus* complex, but is not capable of distinguishing all individual species of this complex.

12.4 PRA-*hsp65* and PRA-ITS

The PRA methodology has been adopted for the identification of mycobacteria in several countries because it is low-cost and easy to perform. Moreover, PRA-*hsp65* profiles of most validated species are available in the PRASITE (http://app.chuv.ch/prasite/index.html). However, overlapping patterns have been reported and multiple patterns within a single species may also occur. Both situations represent the major limitation of PRA (da Silva Rocha, da Costa Leite et al. 1999, Roth, Reischl et al. 2000, Leao, Bernardelli et al. 2005, Martin, Uwizeye et al. 2007, Chimara, Ferrazoli et al. 2008). Indeed, several overlapping patterns and multiple patterns within a species were observed in the present study.

Among the type strains, PRA-*hsp65* was useful to discriminate *M. abscessus* subsp. *abscessus* ATCC 19977^T, *M. immunogenum* ATCC 700505^T and *M. saopaulense* CCUG 66554^T. *M. abscessus* subsp. *bolletii* CCUG 50184^T and *M. massiliense* CCUG 48898^T showed the same restriction profile (*M. abscessus* type 2) as well as *M. chelonae* ATCC 35752^T and *M. salmoniphilum* (*M. chelonae* type 1). Moreover, *M. franklinii* DSM 45524^T showed a restriction profile registered as *M. immunogenum* type 2.

New restriction profiles of PRA-*hsp65* were identified in the present study. All *M. chelonae* isolates showed *M. chelonae* type 1 profile, except for isolates 96-1705 and 96-892 (the representative strain of CHII cluster), which showed a novel restriction profile not registered in PRASITE database – BstEII [bp] (320,130) and HaeIII [bp] (200,55,50). Since their identification was confirmed by ANI analyses as *M. chelonae* (CHAPTER 10), this restriction profile was named *M. chelonae* type 2. However, isolates of genomospecies A and B, also showed this profile, indicating that it can overlap with other species of the *M. chelonae-M. abscessus* complex. Isolate D16Q15, identified by ANI analyses as *M salmoniphilum*, also showed a restriction profile not registered in PRASITE database – BstEII [bp] (320,130) and HaeIII [bp] (200,115, 60, 50), which is different of that of the *M. salmoniphilum* type strain profile. This profile was also observed in isolates of genomospecies C. Among the new profiles identified in this study, only that of *M. saopaulense*– BstEII [bp] (235,210) and HaeIII [bp] (145,60,55,50) – did not overlap with other species of the

M. chelonae-M. abscessus complex. This profile will be named *M. saopaulense* type 1.

Several overlapping patterns were also observed in PRA-ITS. *M. abscessus* subsp. *abscessus* ATCC 19977^T, *M. abscessus* subsp. *bolletii* CCUG 50184^T, *M. massiliense* CCUG 48898^T and *M. franklinii* DSM 45524^T showed the same restriction profile, registered as *M. abscessus* profile, which was also identified in isolates of *M. saopaulense*, *M. franklinii* and genomospecies A and B. *M. salmoniphilum* ATCC 13758^T showed a restriction profile registered as *M. chelonae* type III, which was the same for isolate D16Q15 and isolates of genomospecies C. Moreover, *M. chelonae* isolates showed *M. chelonae* type I or type II restriction profiles. Only *M immunogenum* ATCC 700505^T showed a new PRA-ITS profile – TaqI [bp] (150/125).

Therefore, in general, the discriminative power of PRA-*hsp65* was higher than those of PRA-ITS and the commercial assay GenoType® CM. However, several overlapping patterns were observed, hindering the discrimination of all species of the *M. chelonae-M. abscessus* complex. Since PRA-*hsp65* is less expensive than commercial assays and DNA sequencing, it is still widely used for NTM identification by reference laboratories in low-income countries, such as Brazil. Therefore, the laboratories should be aware of overlapping restriction patterns and multiple patterns among members of *M. chelonae-M. abscessus* complex. Both situations can lead to ambiguous or erroneous species identification. Furthermore, the laboratories should also be aware of the description of new species and their restriction profiles.

Species identification by PRA is based on the patterns included in the PRASITE for PRA-*hsp65* and in the publication of Roth *et al*. (2000) for PRA-ITS (Roth, Reischl et al. 2000). However, the PRASITE is not updated since 2008 and the patterns of the species recently described *M. salmoniphilum*, *M. franklinii* and *M. saopaulense* have not been included. The study of Roth *et al*. was published in 2000 when *M. abscessus* and *M. chelonae* were the only officially accepted species of the *M. chelonae-M. abscessus* complex.

Other problems were faced during the performance of PRA-*hsp65* and PRA-ITS. As they are not automated techniques, they are performed as in house methodologies requiring additional work to standardize all steps, including PCR and electrophoresis conditions. For some isolates, it was necessary to change the concentration of PCR reagents to amplify *hsp65* or ITS. Moreover, the quality of

agarose also influenced the results. To better separate the bands with small bp differences, a high-resolution agarose was used, increasing the costs. Even using it, sometimes the interpretation of digestion profiles was doubtful and the assay needed to be repeated. Therefore, in general the whole process of PRA-*hsp65* and PRA-ITS was time-consuming and labor-intensive.

12.5 DNA sequencing

In the present study, the partial 16S rRNA sequences (1384 bp) of all isolates shared more than 99% identity with the respective sequences of all type strains of the *M. chelonae-M. abscessus* complex (ANNEX 1). The 16S rRNA inability to differentiate closely related species has been described in several studies (Rogall, Wolters et al. 1990, Adekambi, Colson et al. 2003, Adekambi and Drancourt 2004b, Simmon, Brown-Elliott et al. 2011, Joao, Cristovao et al. 2014). However, the use of 16S rRNA sequencing in mycobacteria taxonomic studies is important since it shows the nearest phylogenetic neighbors (Tortoli, Bartoloni et al. 2001).

In particular, regarding the two new species described in this study, partial sequencing of *rpoB* and *hsp65* genes appears to be a good tool for distinguishing *M. franklinii* and *M. saopaulense* from other members of *M. chelonae-M. abscessus* complex.

Over the last decades studies have shown the inaccuracy of a single gene for differentiation of mycobacterial species since particular limitations of each gene have been identified (Macheras, Roux et al. 2009, Dai, Chen et al. 2011). Besides the limited variation in 16S rRNA sequences (Rogall, Wolters et al. 1990, Adekambi, Colson et al. 2003, Adekambi and Drancourt 2004b, Joao, Cristovao et al. 2014), high intra-species variability of *rpoB* sequences has been reported (Adekambi, Colson et al. 2003). Moreover, several studies suggested the occurrence of genetic exchange among the *M. chelonae-M. abscessus* complex members involving housekeeping genes such as *hsp65*, *sodA*, *rpoB*, *cya* and *glpK* genes (Kim, Kook et al. 2008, Viana-Niero, Lima et al. 2008, Zelazny, Root et al. 2009, Macheras, Roux et al. 2011). Macheras *et al*. (2011) demonstrated the occurrence of horizontal transfer of *rpoB* sequences among between *M. abscessus* and *M. massiliense* (Macheras, Roux et al. 2011). Genetic exchange can lead to recombination events between

homologous genes and therefore isolates can show a composition of genes of different members of the *M. chelonae-M. abscessus* complex (Macheras, Roux et al. 2009, Macheras, Roux et al. 2011). As a consequence, the use of a single gene can lead to misidentification at species level.

In the present study, we demonstrated that the use of a single gene could be a pitfall when working with species that have never been described. The analysis of a single gene would lead to a misclassification since high identities are found with other members of *M. chelonae-M. abscessus* complex (ANNEX 1). In fact, this occurs because the members of this complex are closely related. However, when using two or more genes, discordant results were observed, indicating the possibility of being a new species.

Other issue that deserves special attention is that identification based on DNA sequencing is dependent on databases such as GenBank. In general, sequences submitted to databases are not evaluated in terms of quality and sequences with errors, incomplete sequences and sequences with misassigned names can be deposited. As a consequence, erroneous identification can occur when sequences are compared to those deposited in databases. A practical example was the deposit of *rpoB* sequence of *M. massiliense* CCUG 48898. The first deposited sequence contained 5 erroneous nucleotides. The sequence was then corrected and now a second version is available (AY593981.2).

12.6 DNA-DNA Hybridization (DDH)

As previously reported, several difficulties were faced during DDH experiments performance, especially regarding the purity and DNA concentration. As discussed above, it is really hard to achieve high purity and high concentration DNA when working with mycobacteria, since they have a complex cell wall structure. Therefore, several protocols were tested and that described by Pitcher *et al*. (Pitcher, Saunders et al. 1989), which has been widely used for gram positive bacteria, showed the best results. It uses a lysis buffer containing lysozyme and mutanolysine. Lysozyme hydrolyzes the peptidoglycan present in the cell wall and it has been used for DNA extraction of mycobacteria (van Soolingen, de Haas et al. 1994). Mutanolysine cleaves the linkage of peptidoglycan-polysaccharide of the bacterial cell wall. Its use

is not common in DNA extraction protocols for mycobacteria; however, in this study it produced significantly higher DNA concentrations.

Another problem faced in DDH experiments was related to the values of the reciprocal experiments. In some cases they were discordant and the experiments had to be repeated several times.

As DDH was long considered the reference method in taxonomic studies for species delineation (Vandamme 2012), it was performed for the description of the new species, *M. franklinii* and *M. saopaulense*.

DDH experiments were also performed with the type strains of *M. chelonae-M. abscessus* complex. Reciprocal experiments between all type strains were below 70%, except between *M. abscessus* subsp. *abscessus* ATCC 19977^T, *M. abscessus* subsp. *bolletii* CCUG 50184^T and *M. massiliense* CCUG 48898^T, that were above 70%. These results confirmed that the type strains represent distinct species of *M. chelonae-M. abscessus* complex and that strains ATCC 19977^T, CCUG 50184^T and CCUG 48898^T belong to the same species (*M. abscessus*). These results were subsequently used to validate the genome comparisons.

12.7 Whole Genome Sequence Comparison

Pairwise genome comparisons were performed using average nucleotide identity (ANI), tetranucleotide frequency correlation coefficients (TETRA) analysis, genomic signature (delta values), genome-to-genome distance (GGD) calculations, and digital DNA-DNA hybridization (dDDH). All genome comparisons results were presented in CHAPTER 10.

ANI between the type strains of *M. chelonae-M. abscessus* complex were concordant with DDH experiments. These results validated the application of ANI in comparisons using other isolates included in this study.

The isolates identified as *M. chelonae* in the first phase of this study (96-1705, 96-1717, 96-1720, 96-1724, 96-1728, D16Q24, D16R2, D16R3, D16R7, D16R9, D16R10, D16R14, D16R19 and D16R20) yielded ANI values above 95% with ATCC 35752^T and below 95% with the other type strains, confirming that they indeed belong to the species *M. chelonae*. Isolate D16Q15 yielded ANI values above 95% with ATCC 13758^T, showing that it belongs to the species *M. salmoniphilum*. Isolates

D16R27 and D16Q19 yielded ANI values above 95% with DSM 45524^T, indicanting that they belong to the species *M. franklinii*. However the other isolates yielded ANI values below 95% with all type strains indicating a clear separation from the known species of the *M. chelonae-M. abscessus* complex. Among these isolates, three genomospecies were identified: D16Q14, D16Q20, D16R24 and D17A2 (Genomospecies A), 96-892, D16Q13 and D16Q16 (Genomospecies B); and D16R12 and D16R18 (Genomospecies C). The pairwise comparison of isolates in each genomospecies yielded ANI values above 95%, indicating that they belong to the same species.

Discrepant results between ANI and TETRA were observed. All TETRA coefficients, even between pairwise type strains, were above the 0.99 threshold. Moreover, isolates with pairwise ANI values above 95% showed TETRA values above 0.999. TETRA coefficients between pairwise type strains of *M. abscessus* showed values above 0.999 while comparisons between the other type strains were above 0.99 but below 0.999. This finding shows that for closely related species of the *M. chelonae-M. abscessus* complex the TETRA threshold could be higher than 0.99.

ANI has been recognized as one of the most reliable tools to measure genomic relatedness between bacterial strains (Kim, Oh et al. 2014). Richter and Rossello-Mora (2009) showed that TETRA correlates with ANI and assists to decide if a pair of isolates belongs to the same species. However Ngeow *et al*. (2015) also found discrepancies between ANI and TETRA when studying closely related mycobacteria from *M. terrae* complex. TETRA indicated that some strains belonged to the same species while ANI values were all below 95%. This discrepancy was attributed to the influence of environmental factors, such as the acquisition of foreign sequences, which probably lead to a genome diversification without changing the TETRA signature. Indeed, a previous study reported that closely related bacteria present in the same environment have small differences in G+C distributions (Foerstner, von Mering et al. 2005). Considering that the majority of the isolates included in the present study were recovered from water sources, the hypothesis of Ngeow *et al*. (2015) could explain the discrepant results of TETRA. As the members of *M. chelonae-M. abscessus* complex are closely related and live in similar habitats, genome size expansion can occur while TETRA signature is conserved. Further

investigations should be performed to confirm this hypothesis, including the study of mycobacteria isolates that don't belong to *M. chelonae-M. abscessus* complex.

A similar approach, namely genomic signature, was performed. It allowed the calculation of delta differences between genomes. The obtained results were not useful to differentiate the isolates from this study, suggesting that the correlation of frequencies of di- or tetra nucleotides is not applicable for species delimitation of closely related mycobacteria, such as those of the *M. chelonae-M. abscessus* complex.

The results obtained with GGD were in general congruent with the results obtained with ANI. However, some GGD values were above the cutoff value in some cases in which GGD values below the cutoff were expected. Remarkably, *M. abscessus* subsp. *abscessus* ATCC 19977^T, *M. abscessus* subsp. *bolletii* CCUG 50184^T and *M. abscessus* subsp. *massiliense* CCUG 48898^T showed a distance higher than the accepted cutoff (0.0266 to 0.0288 distance values) although the calculated dDDH values were those expected for a single species (75.5% to 77.3%). The same was observed with two groups clustered by ANI: D16Q15 and *M. salmoniphilum* ATCC 13758^T showed 0.0340 distance value; D16Q19, D16R27 and *M. franklinii* DSM 45524^T showed 0.0336 distance values. Both groups had >70%, dDDH values, which were in accordance with ANI values of 96.5%.

The 14 isolates that showed >95% ANI values with *M. chelonae* ATCC 35752^T showed distance values above the cutoff for species differentiation (0.0449 to 0.0458) and <70% dDDH (63.50 to 64.00) with the *M. chelonae* type strain. Similar results were found after comparison of genomes of strains D16R12 and D16R18 (GGD=0.0480; dDDH=62.2%) – the proposed genomospecies C. D16R12 and D16R18 showed ANI values lower than 95.5%, near the threshold value, thus suggesting the occurrence of some sequence divergence between these genomes. These data suggest that a revision of the threshold values for GGD is needed, particularly for species with inner variability, such as *M. abscessus* and other mycobacteria included in this study.

Considering that isolates of this study were recovered from water sources and that infection by members of *M. chelonae-M. abscessus* complex are mainly acquired from environmental exposure including interactions with water (Brown-Elliott and Wallace Jr. 2012b, Donohue, Mistry et al. 2015), the use of genomic tools for

identification should be implemented in the clinical laboratory for better discrimination of these isolates. However, WGS comparison has been scarcely used in the species differentiation of mycobacteria and, therefore, more studies are required to explain the discrepancies found in this study by using different WGS comparative approaches. Probably the current threshold values applied in the procedures used for WGS species delineation should be reevaluated for closely related species of mycobacteria, such as the members of the *M. chelonae-M. abscessus* complex.

As shown in CHAPTER 10, before WGS becomes affordable in the routine laboratory, this study indicates that analysis of *rpoB* gene or concatenated sequences of 16S rRNA, *hsp65, rpoB* and ITS can be useful for the identification of members of *M. chelonae-M. abscessus* complex.

12.8 Impact of the present study

Members of the *M. chelonae-M. abscessus* complex have been isolated from several water sources (Falkinham 2009, van Ingen, Blaak et al. 2010, Gomez-Alvarez, Revetta et al. 2012, Velayati, Farnia et al. 2014, Donohue, Mistry et al. 2015, Petti, Polimeni et al. 2015). As they are disinfectant-resistant, they can resist water treatment entering houses, buildings and hospitals plumbing (Brown-Elliott and Wallace 2002, Primm, Lucero et al. 2004, Donohue, Mistry et al. 2015, Falkinham 2016). Gomes-Alvarez *et al*. (2012) showed that several members of the *M. chelonae-M. abscessus* complex could be isolated from drinking water distribution systems (Gomez-Alvarez, Revetta et al. 2012). Although the majority of the environmental investigations failed to identify the source of infection, it is generally presumed that infections occur after environmental exposure, such as after contact with water (Brown-Elliott and Wallace Jr. 2012b, Donohue, Mistry et al. 2015).

In 2012, an outbreak of infectious keratitis following LASIK occurred in the state of São Paulo, Brazil. Clinical and environmental samples from the ophthalmological facility were collected and sent to our laboratory. The samples were analyzed in parallel with the isolates included in this study. The presence of a unique strain of *M. chelonae* recovered from clinical samples and distilled water was demonstrated. Distilled water was prepared on a daily base in the surgical facility from tap water and kept in plastic reservoirs. This study concluded that the original source of the *M. chelonae* strain was the tap water demonstrating the environmental source of contamination (ANNEX 2). Considering that the majority of the isolates in the present study were recovered from water sources and infections may occur due to environmental exposure, all challenges faced in our study to achieve the identification at species level could be also faced in a clinical laboratory. Furthermore, the methodologies used in our study are applicable in future investigations of infections and outbreaks caused by members of *M. chelonae-M. abscessus* complex.

As demonstrated in this study, WGS is a reliable tool for species identification. However, it is not available in clinical microbiology laboratories yet. Therefore, other methodologies, such as partial DNA sequencing and MALDI-TOF are being used to replace the phenotypic and culture tests that are time consuming and sometimes not

discriminatory enough. The commercial kit GeneXpert® (Cepheid), based on real-time PCR, has been incorporated in clinical laboratories for *M. tuberculosis* identification and rifampicin susceptibility testing, for example. Moreover, reference laboratories are performing partial DNA sequencing to confirm NTM species identification. However, as shown in this study, before WGS becomes affordable in routine laboratories, species identification should not be based on a single methodology. The use of two or more methods makes species identification more accurate and can reveal the presence of species not yet described. As demonstrated, analysis of *rpoB* gene or concatenated sequences of 16S rRNA, *hsp65, rpoB* and ITS are good tools for the correct identification of members of *M. chelonae-M. abscessus* complex, which is really important for patient management.

PART V: CONCLUSIONS AND PERSPECTIVES

12 Conclusions

1. A high phenotypic and genotypic variability in the CHII INNO-LiPA® cluster isolates was confirmed.
2. Biochemical and culture tests were poorly discriminative and none of the phenotypic tests was able to fully discriminate the members of *M. chelonae-M. abscessus* complex.
3. Variability of antimicrobial patterns within members of *M. chelonae-M. abscessus* complex was observed emphasizing the necessity to perform DST in clinical isolates before treatment initiation.
4. GenoType® CM did not allow to distinguish all individual species of the *M. chelonae-M. abscessus* complex.
5. Overlapping and multiple patterns of PRA-*hsp65* and PRA-ITS were observed hindering discrimination of the *M. chelonae-M. abscessus* complex members using these techniques.
6. A novel PRA-*hsp65* pattern, called *M. chelonae* type 2 [BstEII (320 bp,130 bp, HaeIII (200 bp, 55 bp, 50 bp)] was described.
7. The inability of single gene sequence analysis for species identification of members of the *M. chelonae-M. abscessus* complex was reaffirmed.
8. WGS and genome comparisons were useful to determine the taxonomic status of isolates which were not resolved into definite species with conventional tests.
9. Current threshold values applied in comparative genomics using TETRA and delta differences could not be used for separation of closely related members of the *M. chelonae- M. abscessus* complex.
10. Two new species were described and validly named; *M. franklinii* and *M. saopaulense*, and three novel genomospecies were identified.
11. The correct identification of *M. franklinii* and *M. saopaulense* is epidemiologically and clinically important. Both species can cause human diseases. *M. franklinii* caused pulmonary infections; *M. saopaulense* caused eye infections and cervical lymphadenitis. Infections caused by *M. saopaulense* were really difficult to treat; the patients with eye infection had to be submitted to corneal transplants and the patient with lymphadenitis died with disseminated mycobacterial infection. Moereover an important difference in drug susceptibility was demonstrated, as *M. franklinii* is

susceptible to most tested antimicrobials, while *M. saopaulense* is the most drug resistant species withtin the *M. chelonae-M. abscessus* complex.

12. Finally, this study emphasizes that mycobacteria identification should not be based on a single methodology. The use of two or more methodologies makes the species identification more accurate and can also reveal the presence of species not yet described.

13 Perspectives

With the greater accessibility of WGS, bacterial taxonomy has been launched into a new era of genomic taxonomy. The present study showed that WGS was useful to determine the taxonomic status of unknown isolates from the *M. chelonae-M. abscessus* complex and could be used in future investigations of infections and outbreaks caused by members of *M. chelonae-M. abscessus* complex, allowing more precise species classification.

One of the problems faced by genomic taxonomy is the scarcity of genomes deposited in public database for comparative analysis. The raw sequencing data of all type strains and isolates sequenced in the present study, including the three new genomospecies and the two new species, were deposited in NCBI, enlarging the database for future investigations. A comparative analysis of the genomes of all members of *M. chelonae-M. abscessus* complex, could allow the identification of new molecular targets for identification of its members. Moreover, a new line probe assay for accurate species identification within the *M. chelonae-M. abscessus* complex could be developed.

With the increasing performance of analyses based on WGS, additional taxonomic changes such as reclassifications and the description of new species will probably occur in the *M. chelonae-M. abscessus* complex. It is important to highlight that taxonomic changes should be accompanied by a constant evaluation of the methods used for mycobacteria identification at species level in order to ensure proper identification of clinical and environmental isolates.

Finally, the present study evidenced that the identification of mycobacterial species should not be based on a single methodology. The use of two or more methods makes species identification more accurate and can reveal the presence of species not yet described.

ENGLISH ABSTRACT

The methodologies used to delineate bacterial species are changing with the development of new technologies. The *Mycobacterium chelonae-Mycobacterium abscessus* complex is composed of closely related rapidly growing mycobacteria that are ubiquitous in the environment and sometimes responsible for a wide spectrum of opportunistic infections in humans, especially pulmonary infections and localized skin and soft tissue infections. This complex has expanded over the years to include, besides the classical species *M. chelonae* and *M. abscessus*, also *M. immunogenum*, *M. salmoniphilum*, and two subspecies *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii*. Despite all technological advances, the taxonomic relations of members of *M. chelonae-M. abscessus* complex still generate several doubts, due to important similarities in their phenotypic and genotypic characteristics. Considering the importance of correct species identification for understanding the taxonomy of this group and for outbreak detection and therapeutic patient management, the present project aimed at investigating the taxonomic position of a set of *M. chelonae-M. abcessus* complex isolates without conclusive species identification and a set of isolates of the CHII INNO-LiPA® cluster. A polyphasic approach, including phenotypic analyses traditionally used for identification of mycobacteria, drug susceptibility testing, the commercial test Genotype® *Mycobacterium* CM and AS, PCR Restriction Enzyme Analysis of the *hsp65* gene and 16S-23S internal transcribed spacer (ITS), DNA sequencing of the 16S rRNA, *hsp65* and *rpoB* genes and the 16S-23S ITS, DNA-DNA hybridization (DDH), Whole Genome Sequencing (WGS) and genome comparisons were carried out. The previously reported high variability of the CHII INNO-LiPA® cluster was confirmed. Two species were validly named as new members of *M. chelonae-M. abscessus* complex, *M. franklinii* and *M. saopaulense*, and three additional genomospecies were identified. Moreover, genomic analyses confirmed that the type strains of the *M. chelonae-M. abscessus* complex, including the two recently described species (*M. franklinii* and *M. saopaulense*), belong to different species, confirming the current taxonomic classification of this complex in the era of genomic taxonomy. This study illustrates the use of WGS to determine the taxonomic status of isolates which conventional tests fail to identify. Furthermore it emphasizes that

mycobacteria identification should not be performed based only in a single methodology. This study will be useful for future investigations of infections and outbreaks caused by members of *M. chelonae-M. abscessus* complex.

NEDERLANDSE SAMENVATTING

De onderzoeksmethoden die gebruikt worden om bacteriële soorten af te bakenen veranderen met technologische ontwikkelingen. De *Mycobacterium chelonae-Mycobacterium abscessus* groep bevat een aantal nauwverwante snelgroeiende mycobacteriën die zeer algemeen in de omgeving voorkomen en die verantwoordelijk zijn voor een waaier van opportunistische infecties in mensen, waaronder voornamelijk longinfecties, huidinfecties en infecties in zachte weefsels. Deze groep van bacteriën is gedurende de recente jaren uitgebreid en bevat naast de traditionele species *M. chelonae* en *M. abscessus* eveneens *M. immunogenum*, *M. salmoniphilum*, en de twee subspecies *M. abscessus* subsp. *abscessus* en *M. abscessus* subsp. *bolletii*. Ondanks alle technologische vooruitgang is er nog veel onduidelijkheid rond de taxonomische verwantschappen van leden van de *M. chelonae-M. abscessus* groep, en dit omwille van grote gelijkenissen in hun fenotypische en genotypische eigenschappen. Gezien het belang van correcte species identificatie om de taxonomie van deze bacteriën te begrijpen en omwille van de detectie van uitbraken en van therapeutische beslissingen in verband met patiëntbehandeling en -management, stelde het voorliggend project zich tot doel de taxonomische verwantschappen te bepalen van *M. chelonae-M. abcessus* groep isolaten zonder eenduidige species identificatie en van een set van isolaten die behoren tot het CHII INNO-LiPA® cluster. Een polyfasische benadering werd hiervoor toegepast. Deze bevatte fenotypische testen die traditioneel gebruik worden voor de identificatie van mycobacteriën, antibioticumgevoeligheidsbepalingen, de commerciële testsystemen Genotype® *Mycobacterium* CM en AS, PCR restrictie-enzymanalyse van het *hsp65* gen en van de 16S-23S interne spacer (ITS), partiële sequenering van de 16S rRNA, *hsp65* en *rpoB* genen en van de 16S-23S ITS, DNA-DNA hybridisatie en onderzoek van volledige genoomsequenties. De eerder gerapporteerde grote variabiliteit binnen het CHII INNO-LiPA® cluster werd bevestigd. Twee species warden op een geldige manier beschreven als nieuw leden van de *M. chelonae-M. abscessus* groep, namelijk *M. franklinii* en *M. saopaulense*, en drie nieuwe genomospecies werden voor het eerst gerapporteerd. Onze studies van volledige genoomsequenties bevestigden dat de type stammen van de *M. chelonae-M. abscessus* groep, met inbegrip van de nieuwe species *M. franklinii* en

M. saopaulense, effectief verschillende species vertegenwoordigen, wat hun huidige taxonomische status onderschrijft. Ons onderzoek toonde ook aan dat het gebruik van genoomsequenties om de taxonomische status van problematische isolaten te bepalen een haalbare kaart is. Daarenboven benadrukte ons werk dat de identificatie van mycobacteriën beter niet gesteund is op één enkele methodologie. Onze studie zal ten slotte een basis vormen voor verder onderzoek naar infecties en uitbraken veroorzaakt door vertegenwoordigers van de *M. chelonae-M. abscessus* groep.

ANNEX 1

ANNEX 1. Percentage of sequence identity between the isolates and each type strain of *M. chelonae-M. abscessus* complex.

Group	Isolate	16S rRNA (1384 bp)*							*hsp65* (401 bp)							*rpoB* (711 bp)#							ITS (214 bp)§						
		1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7
1	**8223**	**100**	**100**	**100**	99.7	99.5	99.3	99.7	**100**	98.5	98.8	92.8	91.8	90.8	92.5	**100**	95.7	96.5	93.5	94.9	92.4	93.9	**100**	**100**	99.5	91.1	89.7	93.0	93.5
2	96-1705	**99.8**	**99.8**	**99.8**	99.6	99.3	99.6	99.6	93.3	92.8	93.0	**98.8**	96.0	96.0	97.0	93.8	95.6	95.9	**99.2**	97.5	96.1	97.0	91.6	91.6	91.6	**99.1**	91.1	94.4	94.9
	96-1712	**99.9**	**99.9**	**99.9**	99.8	99.4	99.4	99.8	92.8	92.3	92.5	**99.5**	94.8	95.8	95.8	93.5	95.5	95.8	**99.7**	96.9	96.3	97.0	91.6	91.6	91.6	**99.5**	91.6	94.4	94.9
	96-1717	**99.9**	**99.9**	**99.9**	99.8	99.4	99.4	99.8	93.0	92.5	92.8	**99.8**	95.0	96.0	96.0	93.5	95.5	95.8	**99.7**	97.2	96.3	97.0	91.1	91.1	91.1	**99.1**	91.1	94.9	94.4
	96-1720	**99.9**	**99.9**	**99.9**	99.8	99.4	99.4	99.8	93.0	92.5	92.8	**99.8**	95.0	96.0	96.0	93.5	95.5	95.8	**99.7**	97.2	96.3	97.0	91.1	91.1	91.1	**99.1**	91.1	94.9	94.4
	96-1724	**99.9**	**99.9**	**99.9**	99.8	99.4	99.4	99.8	93.0	92.5	92.8	**99.8**	95.0	96.0	96.0	93.4	95.4	95.6	**99.6**	97.3	96.5	96.9	90.7	90.7	90.7	**99.5**	91.6	95.3	94.9
	96-1728	**99.9**	**99.9**	**99.9**	99.8	99.4	99.4	99.8	93.0	92.5	92.8	**99.8**	95.0	96.0	96.0	93.3	95.2	95.5	**99.4**	97.2	96.3	96.8	91.1	91.1	91.1	**99.1**	91.1	94.9	94.4
	96-443	**99.9**	**99.9**	**99.9**	99.8	99.4	99.4	99.8	93.0	92.5	92.8	**99.8**	95.0	96.0	96.0	93.5	95.5	95.8	**99.7**	97.2	96.3	97.0	90.7	90.7	90.7	**98.6**	90.7	94.4	93.9
	D16Q24	**99.9**	**99.9**	**99.9**	99.8	99.4	99.4	99.8	93.0	92.5	92.8	**99.8**	95.0	96.0	96.0	93.5	95.5	95.8	**99.7**	97.2	96.3	97.0	90.7	90.7	90.7	**98.6**	90.7	94.4	93.9
	D16R2	**99.9**	**99.9**	**99.9**	99.8	99.4	99.4	99.8	93.0	92.5	92.8	**99.8**	95.0	96.0	96.0	93.5	95.5	95.8	**99.7**	97.2	96.3	97.0	90.7	90.7	90.7	**98.6**	90.7	94.4	93.9
	D16R3	**99.9**	**99.9**	**99.9**	99.8	99.4	99.4	99.8	93.0	92.5	92.8	**99.8**	95.0	96.0	96.0	93.5	95.5	95.8	**99.7**	97.2	96.3	97.0	90.7	90.7	90.7	**98.6**	90.7	94.4	93.9
	D16R4	99.7	99.7	99.7	**100**	99.3	99.6	**100**	92.8	92.3	92.5	**99.5**	94.8	95.8	95.8	93.5	95.5	95.8	**99.9**	97.2	96.3	97.0	91.1	91.1	91.1	**100**	92.1	94.9	95.3
	D16R7	**99.9**	**99.9**	**99.9**	99.8	99.4	99.4	99.8	93.0	92.5	92.8	**99.8**	95.0	96.0	96.0	93.5	95.5	95.8	**99.7**	97.2	96.3	97.0	90.7	90.7	90.7	**98.6**	90.7	94.4	93.9
	D16R9	**99.9**	**99.9**	**99.9**	99.8	99.4	99.4	99.8	93.0	92.5	92.8	**99.8**	95.0	96.0	96.0	93.4	95.4	95.6	**99.6**	97.3	96.5	96.9	90.7	90.7	90.7	**99.5**	91.6	95.3	94.9
	D16R10	**99.9**	**99.9**	**99.9**	99.8	99.4	99.4	99.8	93.0	92.5	92.8	**99.8**	95.0	96.0	96.0	93.4	95.4	95.6	**99.6**	97.3	96.5	96.9	90.7	90.7	90.7	**99.5**	91.6	95.3	94.9
	D16R14	**99.9**	**99.9**	**99.9**	99.8	99.4	99.4	99.8	93.0	92.5	92.8	**99.8**	95.0	96.0	96.0	93.5	95.5	95.8	**99.7**	97.2	96.3	97.1	90.7	90.7	90.7	**98.6**	90.7	94.4	93.9
	D16R19	**99.9**	**99.9**	**99.9**	99.8	99.4	99.4	99.8	93.0	92.5	92.8	**99.8**	95.0	96.0	96.0	93.5	95.5	95.8	**99.7**	97.2	96.3	97.1	90.7	90.7	90.7	**98.6**	90.7	94.4	93.9
	D16R20	**99.9**	**99.9**	**99.9**	99.8	99.4	99.4	99.8	93.0	92.5	92.8	**99.8**	95.0	96.0	96.0	93.5	95.5	95.8	**99.7**	97.2	96.3	97.1	90.7	90.7	90.7	**98.6**	90.7	94.4	93.9
3	**6410**	99.5	99.5	99.5	99.3	**100**	98.9	99.3	92.3	92.3	92.5	95.3	**99.0**	97.3	**99.0**	95.6	97.2	98.2	97.0	**99.3**	95.5	96.9	88.8	88.8	88.3	**91.1**	**91.1**	89.3	90.2
4A	**96-892**	99.6	99.6	99.6	**99.9**	99.2	99.8	**99.9**	91.5	92.0	92.3	95.8	96.8	97.0	**97.5**	93.4	95.1	95.6	**97.9**	96.5	96.2	96.8	93.9	93.9	93.5	95.8	90.2	98.1	**98.6**
	D16Q13	99.6	99.6	99.6	**99.9**	99.2	99.8	**99.9**	91.5	92.0	92.3	95.8	96.8	97.0	**97.5**	93.4	95.1	95.6	**97.9**	96.5	96.2	96.8	93.9	93.9	93.5	95.8	90.2	98.1	**98.6**
	D16Q16	99.5	99.5	99.5	**99.8**	99.1	99.7	**99.8**	91.5	92.0	92.3	95.8	96.8	97.0	**97.5**	93.4	95.1	95.6	**97.9**	96.5	96.2	96.8	93.9	93.9	93.5	95.8	90.2	98.1	**98.6**
4B	D16Q14	99.6	99.6	99.6	**99.9**	99.2	99.8	**99.9**	91.3	91.8	92.0	96.0	96.8	**98.0**	97.3	93.0	94.4	94.9	96.5	95.5	95.4	**97.3**	93.0	93.0	92.5	94.4	89.7	**97.2**	96.7
	D16Q20	99.6	99.6	99.6	**99.9**	99.2	99.8	**99.9**	91.3	91.8	92.0	96.0	96.8	**98.0**	97.3	93.0	94.4	94.9	96.5	95.5	95.4	**97.3**	93.0	93.0	92.5	94.4	89.7	**97.2**	96.7
	D16R24	99.6	99.6	99.6	**99.9**	99.2	99.8	**99.9**	91.3	91.8	92.0	96.0	96.8	**98.0**	97.3	93.0	94.4	94.9	96.5	95.5	95.4	**97.3**	93.0	93.0	92.5	94.4	89.7	**97.2**	96.7
	D17A2	99.6	99.6	99.6	**99.9**	99.2	99.8	**99.9**	91.3	91.8	92.0	96.0	96.8	**98.0**	97.3	93.0	94.4	94.9	96.5	95.5	95.4	**97.3**	93.0	93.0	92.5	94.4	89.7	**97.2**	96.7
5	D16Q15	99.3	99.3	99.3	99.6	99.0	**100**	99.6	92.0	92.0	92.3	96.9	96.8	**97.8**	**97.8**	91.8	93.2	93.8	95.9	95.2	**99.0**	95.1	92.5	92.5	92.1	93.9	88.3	**97.7**	97.2
	D16R12	99.4	99.4	99.4	99.7	99.1	**99.8**	99.7	92.0	92.0	92.3	96.9	96.8	**97.8**	**97.8**	93.2	94.2	94.9	96.3	95.8	**97.7**	95.6	93.9	93.9	93.5	95.8	90.2	**99.1**	98.6
	D16R18	99.4	99.4	99.4	99.7	99.1	**99.8**	99.7	92.3	92.3	92.5	95.8	96.5	97.5	**97.8**	93.0	94.1	94.7	96.3	95.5	**98.2**	95.4	93.5	93.5	93.0	95.3	89.7	**98.6**	98.1
6	D16Q19	99.7	99.7	99.7	**100**	99.3	99.6	**100**	92.8	92.3	93.0	96.0	98.2	97.2	**99.7**	94.0	95.2	95.9	96.8	96.5	96.2	**98.6**	93.5	93.5	96.3	93.0	89.7	97.7	**99.1**
	D16R27	99.7	99.7	99.7	**100**	99.3	99.6	**100**	92.8	92.3	93.0	96.0	98.2	97.2	**99.7**	94.0	95.2	95.9	96.8	96.5	96.2	**98.6**	93.5	93.5	96.3	93.0	89.7	97.7	**99.1**
7	EPM 10906	**99.9**	**99.9**	**99.9**	99.8	99.4	99.4	99.8	**97.8**	96.3	96.5	93.3	92.5	92.0	93.3	94.5	95.6	96.6	**97.5**	97.3	95.6	97.2	96.3	96.3	95.8	94.9	89.7	96.7	**97.2**
	EPM 10695	**99.9**	**99.9**	**99.9**	99.8	99.4	99.4	99.8	**97.8**	96.3	96.5	93.3	92.5	92.0	93.3	94.5	95.6	96.6	**97.5**	97.3	95.6	97.2	96.3	96.3	95.8	94.9	89.7	96.7	**97.2**
	IAL 3785	**99.9**	**99.9**	**99.9**	99.8	99.4	99.4	99.8	**97.8**	96.3	96.5	93.3	92.5	92.0	93.3	94.4	95.5	96.5	**97.3**	97.2	95.5	97.0	95.3	95.3	94.9	93.9	88.8	95.8	**96.3**
	JAN1	**99.9**	**99.9**	**99.9**	99.8	99.4	99.4	99.8	**97.8**	96.3	96.5	93.3	92.5	92.0	93.3	94.5	95.8	96.5	**97.6**	97.2	95.8	97.3	95.8	95.8	95.3	94.4	89.3	96.3	**96.7**
	JAN2	**99.9**	**99.9**	**99.9**	99.8	99.4	99.4	99.8	**97.8**	96.3	96.5	93.3	92.5	92.0	93.3	94.5	95.8	96.5	**97.6**	97.2	95.8	97.3	95.8	95.8	95.3	94.4	89.3	96.3	**96.7**

(1) *M. abscessus* subsp. *abscessus*, (2) *M. abscessus* subsp. *bolletii*, (3) *M. massiliense*, (4) *M. chelonae*, (5) *M. immunogenum*, (6) *M. salmoniphilum*, (7) *"M. franklinii"*

*The 16S rRNA partial sequence of 6410 and *M. immunogenum* DSM 45595^T has 1385 bp due to a 1 bp insertion after nucleotide 1354.

#The *rpoB* partial sequences of the isolates of groups 4B and 6, and *M. franklinii* DSM 45524^T have 726 bp. The sequences of the other type strains of the *M. chelonae-M. abscessus* complex have 711 bp. This difference is due to two insertions, one with 6 bp, between nucleotides 409 and 410 and the other with 9 bp, between nucleotides 424 and 425 of the 711 bp sequence.

§The ITS sequences of the groups 4, 5 and 6, *M. franklinii* DSM 45524^T and *M. salmoniphilum* ATCC 13758^T have 214 bp, those of the isolates of groups 1, 2 and 7, *M. abscessus* subsp. *abscessus* CIP 104536^T, *M. abscessus* subsp. *bolletii* CCUG *50184*^{T}, *M. massiliense* CCUG 48898^T and *M. chelonae* ATCC 35752^T have 215 bp (1 bp insertion after nucleotide 20), that of 6410 has 217 bp (1 bp insertion after nucleotide 20 and 2 bp insertion between nucleotides 171 and 172) and that of *M. immunogenum* CIP 106684^T has 225 bp (1 bp insertion after nucleotide 20 and 10 bp insertion between nucleotides 90 and 91).

ANNEX 2

ANNEX 2. Identification of infection sources of an outbreak of post-LASIK keratitis caused by *Mycobacterium chelonae*

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ABSTRACT

Microbial keratitis is a rare complication of refractive surgery, especially seen in laser-assisted in situ keratomileusis (LASIK). Nontuberculous Mycobacteria (NTM), in particular *Mycobacterium chelonae,* are frequent agents of sporadic cases and outbreaks of post-LASIK NTM keratitis. Environmental investigation to identify the source of post-LASIK keratitis has been rarely reported. In this work we described the environment and microbiological investigation of an ophthalmological surgical facility after the occurrence of an outbreak of infections by NTM following LASIK. Using a combination of microbiological and molecular methods, including culture before and after decontamination procedures, identification of the isolated microorganisms and drug susceptibility testing and pulsed field gel electrophoresis typing of isolated NTM, we demonstrated the presence of a unique strain of *M. chelonae* causing the outbreak that was associated with a common environmental source of contamination, the distilled water used in the ophthalmological facility where the surgeries were performed. We concluded that the use of tap water distilled locally represents a great risk of infection in ophthalmologic surgical facilities. Special care should be taken in rinsing surgical instruments even through the steamer equipment and in ensuring that the water supply, air filters, and sterilizing facilities in the laser center are free of contamination to reduce the probability of outbreaks. Finally, technical aspects of the surgical procedure need to be optimized because they can have an impact in the occurrence of infection.

INTRODUCTION

Nontuberculous mycobacteria (NTM) are widely distributed in the environment, especially in fresh water sources (rivers, lakes, tap water) and can also contaminate medical reagents, materials and equipment (Falkinham 2009). These organisms have emerged in the last years as an important cause of hard-to-treat postoperative infections, especially following plastic and ophthalmologic surgeries, as well as other invasive medical procedures such as mesotherapy (Wallace, Brown et al. 1998; Freitas, Alvarenga et al. 2003; Viana-Niero, Lima et al. 2008).

Microbial keratitis is a serious ocular infection that develops in eyes with predisposing risk factors such as trauma and corneal surgeries. It is a rare complication of refractive surgery, especially seen in laser-assisted in situ keratomileusis (LASIK), a surgical technique widely used for correction of refractive errors. The LASIK procedure involves the creation of a thin-hinged flat on the front of the cornea using a microkeratome. The flap is lifted during surgery for laser reshaping of the eye and is then replaced to restore the corneal structure. The flap-stroma interface may confine organisms that were eventually carried over to the surgical site during the procedure. The compromised penetration of prophylactic antibiotics in the corneal stroma, associated with the anti-inflammatory effects of steroids, frequently used postoperatively, can contribute to the development of infectious keratitis following this surgery (Paschal, Holland et al. 1992; Moorthy, Valluri et al. 2012).

According to the American Society of Cataract and Refractive Surgery (ASCRS), the most frequent agents of infectious keratitis after LASIK are Gram positive bacteria and nontuberculous mycobacteria (NTM), in particular *Staphylococcus* spp. and *Mycobacterium chelonae,* respectively (Solomon, Donnenfeld et al. 2011). Other fungi and bacteria, such as *Candida*, *Fusarium*, *Aspergillus*, *Streptococcus*, *Pseudomonas*, and *Nocardia*, have also been reported in LASIK-associated corneal infections (Holmes, Bond et al. 2002; Lifshitz, Levy et al. 2005; Feizi, Jadidi et al. 2007; Chen, Tsai et al. 2009; Garg, Chaurasia et al. 2010; Sharma, Jindal et al. 2012; Homa, Shobana et al. 2013).

In this work we described the environment and microbiological investigation of an ophthalmological surgical facility after the occurrence of an outbreak of infections by NTM following LASIK.

MATERIAL AND METHODS

Outbreak characterization

Between December 2011 and February 2012, eight patients (15 eyes) developed keratitis after laser in situ keratomileusis (LASIK) performed in a single clinic in the state of Sao Paulo, Brazil. Samples from seven of the eight patients were obtained with a Kimura spatula either by direct scrapping of the cornea or by lifting the flap. Specimens were submitted to the Central Clinical Laboratory, Hospital Sao Paulo, Federal University of Sao Paulo (UNIFESP), Brazil and were evaluated by direct microscopy (Gram, Giemsa and Ziehl Neelsen staining) and cultivated on defibrinated sheep blood, chocolate, Sabouraud dextrose agar plates, thioglycolate broth and Löwenstein-Jensen slants (Probac do Brasil, São Paulo, Brazil). Blood and chocolate agar plates were incubated at 37°C for 1 to 7 days in ambient atmosphere and 5% CO_2. Löwenstein-Jensen and Sabouraud agar were incubated at 30°C and ambient temperature, respectively, for up to 30 days. Cultures were examined daily for evaluation of growth. Aerobic bacteria were identified by using the Phoenix automated system (Becton and Dickson). Nontuberculous mycobacteria were identified as described later in this section.

Microbiological investigation of the surgical facility

Following the outbreak, in April 2012 an environmental investigation of the surgical facility was performed. Samples of tap water, water from the reservoir of the bench top distiller equipment, from the portable steamer used to clean the microkeratome and from the STATIM 2000 Cassette Autoclave (Scican, Canonsburg, PA, USA) were collected. Imprints of microkeratome rings and other recently used surgical instruments were performed on Middlebrook 7H10-OADC (oleic acid-albumin-dextrose-catalase) (Becton, Dickinson and Company, BD, Franklin Lakes, NJ, USA) agar plates. Samples of antiseptic solutions, microkeratome blades and

cannulas were inoculated directly into Middlebrook 7H9-OADC liquid medium (Becton Dickinson and Co) without previous decontamination (Table 1).

All samples were collected in sterile flasks. Water samples were sent to the mycobacterial laboratory at the Department of Biological Sciences, Federal University of Sao Paulo (UNIFESP), where volumes higher than 100 mL were concentrated by vacuum filtration through 0.45 µm pore size nylon membranes (EMD Millipore, Billerica, MA, USA) and samples with lower volumes were concentrated by centrifugation at 3,000 *g* for 20 min. Concentrated samples were split in two. One half was sent to the Central Laboratory at Hospital São Paulo and cultured on defibrinated sheep blood agar, chocolate agar, and Sabouraud dextrose agar plates as described above. Aerobic bacteria were identified by using the Phoenix automated system (Becton and Dickson) and yeast isolates were identified by the API® ID 32C (bioMérieux). The second half of the samples was decontaminated with 0.05% cetylpyridinium chloride (CPC) (LabSynth do Brasil, Diadema, Brazil) and inoculated on Löwenstein-Jensen slants, Middlebrook 7H10-OADC plates and Middlebrook 7H10-OADC-PANTA (BD and Co) plates (PANTA final concentration: 40 U/ml polymyxin, 4 µg/ml amphotericin B, 16 µg/ml nalidixic acid, 4 µg/ml trimethoprim and 4 µg/ml azlocillin). Duplicate plates were incubated at 30°C and 37°C. Cultures were observed on a daily base until colonies were observed for a maximum of 60 days (Neumann, Schulze-Robbecke et al. 1997; Radomski, Cambau et al. 2010).

Identification of Nontuberculous Mycobacteria

All recovered colonies were initially visualized by microscopy after Ziehl-Neelsen staining. Acid-fast bacilli (AFB) colonies were then identified by evaluation of phenotypic characteristics (growth rate and pigment production) and by PCR-Restriction-Enzyme Analysis (PRA-*hsp*65) according to the protocol described by Chimara *et al*. (Chimara, Ferrazoli et al. 2008). Briefly, a 441-bp fragment of the *hsp65* gene was amplified with primers TB11 (5´ACCAACGATGGTGTGTCCAT) and TB12 (5´CTTGTCGAACCGCATACCCT). Amplicons were digested separately with BstEII (Promega) and HaeIII (Invitrogen). Digestion products were visualized after electrophoresis in 3% agarose gels and digestion fragment sizes were estimated by visual analysis and using the BioNumerics v. 7.5 program (Applied Maths, Sint-Martens-Latem, Belgium). The obtained digestion patterns were compared to those

present at the PRASITE database (http://app.chuv.ch/prasite/index.html). Final identification of AFB isolates was obtained by DNA sequencing of *hsp65* and *rpoB* genes, 16S rRNA and the 16S-23S internal transcribed spacer (ITS). The primer pairs used for amplification and sequencing of these targets are depicted in Table 2. The sequences were edited using the BioEdit program (v. 7.1.9) and compared to sequences deposited in the GenBank database using the basic local alignment tool (BLAST) (URL: http://www.ncbi.nlm.nih.gov/BLAST). The obtained sequences were deposited in GenBank under accession numbers KT779779 to KT779786.

Table 2. Primers used for amplification and sequencing of 16S rRNA, rpoB, *hsp65* and the 16S-23S internal transcribed spacer (ITS).

Genes	Primers	Reference
16S rRNA	16S-27F (5'-AGAGTTTGATCCTGGCTC AG-3')	(Harmsen, Dostal et al. 2003)
	800F (5'-CAGGATTAGATACCCTGGTAG-3')	(Adekambi and Drancourt 2004b)
	16S-907R (5'-CGTCAATTCCTTTGAGT TT-3')	(Harmsen, Dostal et al. 2003)
	16S-1492 (5'-ACGGCTACCTTGTTACG ACT T-3')	(Gomila, Ramirez et al. 2007)
	23S1R (5'-CCCAAAGCCTATATATTCAGC-3')	(Matsumoto, Chimara et al. 2012)
rpoB	MYCOF1 (5'-TCCGATGAGGTGCTGGCAGA-3')	(Macheras, Roux et al. 2011)
	MYCOR2 (5'-ACTTGATGGTCAACAGCT CC-3')	
hsp65	hsp667F- (5'-GGCCAAGACAATTGCGTACG-3')	(Selvaraju, Khan et al. 2005)
	hsp667R- (5'-GGA GCTGACCAGCAGGATG-3')	
ITS	Sp1 (5'-ACCTCCTTTCTAAGGAGCACC-3')	(Roth, Reischl et al. 2000)
	23S1R (5'-CCCAAAGCCTATATATTCAGC-3')	(Matsumoto, Chimara et al. 2012)

Molecular typing by pulsed-field gel electrophoresis (PFGE)

Mycobacteria obtained from patients and from environmental sources were typed by PFGE, as described by Matsumoto *et al*. (Matsumoto, Chimara et al. 2011). Briefly, single colonies were cultivated in Mueller Hinton broth (Oxoid, Hampshire, UK) supplemented with 0.05% of Tween 80. Plugs of bacterial cells were prepared in 1% low-melt preparative agarose (Bio Rad Laboratories, Hercules, CA, USA). DNA was isolated and digested with 30 U DraI (Promega, Madison, WI, USA) at 37^{o}C overight. Plugs were loaded into 1% Pulsed Field Certified Agarose (Bio Rad) in 0.5X TBE buffer (45 mM Tris-HCl, 45 mM boric acid and 1 mM EDTA). Electrophoresis was carried out in a CHEF-DR III System (Bio Rad) at 14°C for 21 h at 6V/cm, with a switch time of 1.6 to 21.3 s. Lambda Ladder PFG Marker (New England Biolabs, Ipswich, MA, USA) was used as the molecular standard. PFGE gel images were analyzed and dendrograms of PFGE patterns were produced with the BioNumerics v.

7.5 program (Applied Maths) using the band-based Dice UPGMA method based on 1.5% optimization and position tolerance.

Antimicrobial susceptibility testing

The minimal inhibitory concentration (MIC) for amicacin (AMK), ciprofloxacin (CIP), clarithromycin (CLR), doxycycline (DXC), cefoxitin (CTX), tobramycin (TOB) minocycline (MIN), moxifloxacin (MOX), gatifloxacin (GAT), linezolid (LIN), imipenen (IMI) and trimethoprim-sulfamethoxazole (SXT) was determined by the broth microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2011). *Mycobacterium chelonae* isolates from two patients and from the distilled water of the surgical facility were cultivated in cation-adjusted Mueller-Hinton broth (Oxoid) for 3 to 5 days at 30ºC. Bacterial suspensions were adjusted to 0.5 McFarland standard and diluted to achieve a final inoculum of approximately $5x10^5$ CFU/mL. The following drug concentrations were tested: CLR, IMI and TOB: 0.5 to 64 μg/ml; CIP, DXC, GAT, MOX and MIN: 0.25 to 32 μg/ml; AMK and CTX: 4-512 μg/ml, STX: 0.25/4.5 to 32/608. Quality control was performed by testing *Staphylococcus aureus* ATCC 29213 strain.

RESULTS

The initial microbiologic investigation of the patients' isolates revealed that *M. chelonae* was the causative agent in four patients. Coagulase negative staphylococci were isolated from eye samples of three patients and *Serratia* from one and were considered to be contaminants as only few colonies were detected.

At the surgical facility, samples of tap water and distilled water from the storage tank and from the reservoir of the portable steamer contained acid-fast bacilli. Distilled water samples also contained Gram-negative bacteria and yeasts. Cultures of the imprints from the microkeratome rings and other surgical instruments, which were rinsed with distilled water after surgical procedures, yielded Gram-negative bacteria growth (**Table 1**).

Table 1: Samples from the surgical facility analyzed in this work.

Environmental samples	volume or quantity	processing	culture	AFB***	non-AFB	identification
tap water	500 mL	vacuum filtration (0.45 µm pore membranes)	*	2 colonies	-	*Mycobacterium mucogenicum*
			**	-	-	-
water distilled in the same day	500 mL	vacuum filtration (0.45 µm pore membranes)	*	5 colonies	-	*Mycobacterium chelonae*
			**	-	red colony	*Methylbacterium extorquens*
distilled water (reservoir of the portable steamer)	8 mL	centrifugation at 3,000 *g* for 20 min	*	2 colonies	-	*Mycobacterium chelonae*
			**	-	different colonies	*Burkholderia cepacia, Burkholderia gladioli* and *Candida guilhermondis*
distilled water (cassette autoclave)	10 mL	centrifugation at 3,000 *g* for 20 min	*	-	-	-
			**	-	different colonies	*Burkholderia cepacia, Sphingomonas paucimobilis* and *Candida guilhermondis*
microkeratome rings	imprints	-	7H10-OADC	-	different colonies	*Kingella kingae* and *Methylobacterium extorquens*
other surgical instruments	imprints	-	7H10-OADC	-	different colonies	*Pseudomonas oryzihabitans*
microkeratome blades	recently used	-	7H9-OADC	-	-	-
cannulas	recently used	-	7H9-OADC	-	-	-
Cetrimide solution (open for 15 days)	3 mL	-	7H9-OADC	-	-	-
Povidine iodine solution (open for 15 days)	0.6 mL	-	7H9-OADC	-	-	-

* Lowenstein-Jensen, 7H10-OADC, 7H10-OADC-Panta (after decontamination with cetylpiridinium chloride)
**defibrinated sheep blood agar, chocolate agar, and Sabouraud dextrose agar plates
*** AFB: Acid-Fast Bacilli

All isolated mycobacteria were non-pigmented rapid growers. Isolates from the distilled water and from the portable steamer containers were identified as *M. chelonae* and the isolate from tap water was identified as *Mycobacterium mucogenicum*. One of the patients' isolates and one isolate from the distilled water tank were identified by sequencing. The corresponding sequences of *hsp65* and *rpoB* genes, 16S rRNA and ITS of both isolates showed 100% similarity and showed highest similarities with the corresponding sequences of *M. chelonae* type strain deposited in the GenBank by BLAST analysis (Table 3).

Table 3. Percent similarity of the sequences obtained with the isolate from the distilled water and the corresponding sequences of the species of the *M. chelonae-M. abscessus* complex.

Gene	*M. chelonae-M. abscessus* type strain	GenBank accession number	% similarity
hsp65 **(401 pb)**	*M. abscessus* CIP 104536^{T}	AY458075	93,02%
	M. chelonae CIP 104535^{T}	AY458074	99,75%
	M. abscessus subsp. *bolletii* CIP 108541^{T}	AY859675	92,52%
	M. franklinii DSM 45524^{T}	KM392059	95,84%
	M. immunogenum CIP 106684^{T}	AY458081	95,01%
	M. massiliense CCUG 48898	AY596465	92,77%
	M. salmoniphilum ATCC 13758^{T}	DQ866777	96,01%
rpoB **(711 pb)**	*M. abscessus* CIP 104536^{T}	AY147164	93,53%
	M. chelonae CIP 104535^{T}	AY147163	99,86%
	M. abscessus subsp. *bolletii* CIP 108541^{T}	AY859692	95,50%
	M. franklinii DSM 45524^{T}	KM392056	94,97%
	M. immunogenum CIP 106684^{T}	AY262739	97,19%
	M. massiliense CCUG 48898	AY593981.2	95,78%
	M. salmoniphilum ATCC 13758^{T}	KM392056	96,62%
ITS **(215 pb)**	*M. abscessus* CIP 104536^{T}	AY593976	90,23%
	M. chelonae ATCC 35752^{T}	AY498739	98,60%
	M. abscessus subsp. *bolletii* CCUG 50184^{T}	GU143888.2	90,23%
	M. franklinii DSM 45524^{T}	HQ153093	93,49%
	M. immunogenum CIP 106684^{T}	AY593977	86,67%
	M. massiliense CCUG 48898	AY593978	89,81%
	M. salmoniphilum ATCC 13758^{T}	DQ866768	94,42%
16S **(1384 pb)**	*M. abscessus* CIP 104536^{T}	AY457071	99,93%
	M. chelonae CIP 104535^{T}	AY457072	100,00%
	M. abscessus subsp. *bolletii* CIP 108541^{T}	AY859681	99,93%
	M. franklinii DSM 45524^{T}	HQ153090	99,78%
	M. immunogenum DSM 45595^{T}	HE654001	99,35%
	M. massiliense CCUG 48898	NR_043002	99,93%
	M. salmoniphilum ATCC 13758^{T}	NR_043989	99,42%

Gram negative bacteria isolated at the laser facility included *Burkholderia cepacia, Burkholderia gladioli, Sphingomonas paucimobilis, Methylbacterium extorquens, Pseudomonas oryzihabitants* and *Kingella kingae. Candida guilhermondis* was isolated from the portable steamer and from water collected from the STATIM autoclave reservoir (Table 1).

M. chelonae isolates obtained from four patients and from the distilled water storage tank (5 colonies) and the portable steamer reservoir (2 colonies) showed

indistinguishable PFGE patterns, which were different from the PFGE pattern of the *M. mucogenicum* isolate (**Figure 1**).

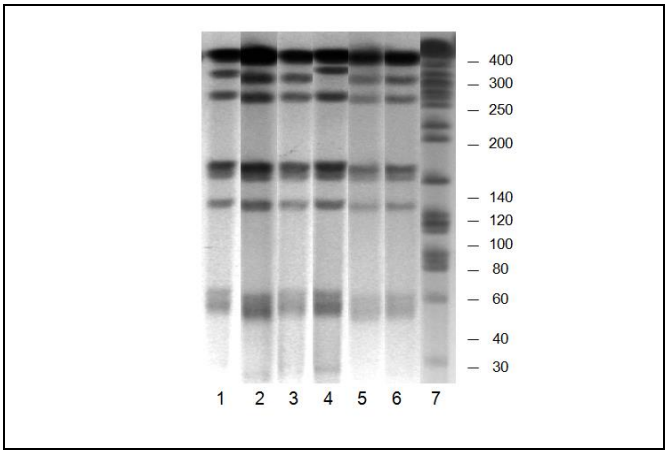

Figure 1: Pulsed field gel electrophoresis patterns of isolates from patients and environmental isolates collected at the surgical facility. 1 - 4: patient's isolates (*M. chelonae*); 5: isolate from the distilled water tank (*M. chelonae*); 6: isolate from the portable steamer (*M. chelonae*); 7: tap water isolate (*M. mucogenicum*); at right: molecular markers (kb)

The antimicrobial susceptibility profile determined by broth microdilution with two patients' isolates and one isolate from the distilled water reservoir are displayed in **Table 4**. The isolates were highly resistant to antibiotics most commonly used for treatment of NTM infections. Among fluoroquinolones, which are frequently used for prophylaxis and treatment of ocular infections, isolates were either intermediate or resistant to ciprofloxacin, moxifloxacin (MIC ranging from 2 to 16 µg/ml) and gatifloxacin (MIC >16 µg/ml). Isolates demonstrated high-level resistance to doxycycline (MIC >32 µg/ml), cefoxitin (MIC ≥ 256 µg/ml) and trimethoprim/sulfamethoxazole (MIC 8/152 µg/ml). Aminoglycosides were not fully active against the isolates, with MIC raging from 4 to 8 µg/ml for tobramycin (intermediate and resistant) and from 8 to 32 µg/ml for amikacin (MIC 8 µg/ml susceptible, but close to the breakpoint and 32 µg/ml intermediate). Isolates were susceptible only to linezolid, with MIC values close to the breakpoint (8 µg/ml), and clarithromycin (MIC ≤0.5 µg/ml), which was the antimicrobial agent showing the highest in vitro activity against these *M. chelonae* isolates.

Table 4: Antimicrobial susceptibility of one isolate from distilled water from the clinic and two patients.

	water from the distilled water tank		Patient 1		Patient 2	
	MIC (µg/ml)	susceptibility[a]	MIC (µg/ml)	susceptibility	MIC (µg/ml)	susceptibility
AMK	32	I	32	I	8	S
CIP	8	R	16	R	2	I
CLR	≤0.5	S	≤0.5	S	<0.5	S
DXC	>32	R	>32	R	>64	R
CTX	512	R	512	R	256	R
TOB	4	I	8	R	4	I
MIN	8	NA	8	NA	32	NA
MOX	2	I	8	R	16	R
GAT	ND[b]	-	>16	NA	>16	NA
LIN	ND	-	8	S	8	S
IMI	ND	-	32	R	32	R
SXT	ND	-	8/152	R	8/152	R

[a] interpretation of MIC results according to CLSI guidelines. S: susceptible; I: intermediate; R: resistant; NA: not available
[b] ND: not done

DISCUSSION

Using a combination of microbiological and molecular typing methods, we demonstrated the presence of a unique strain of *M. chelonae* causing an outbreak of infectious keratitis following LASIK surgery that was associated with a common environmental source of contamination, the distilled water used in the ophthalmological facility where the surgeries were performed.

The first case of post-LASIK keratitis caused by *M. chelonae* was described in 1998 and it was an unilateral infection after simultaneous bilateral surgery (Reviglio, Rodriguez et al. 1998). The authors described the use of the same microkeratome blade in both eyes operated simultaneously and suggested that bacterial inoculation occurred intraoperatively following the corneal incision. Since then, reports of sporadic cases and outbreaks of post-LASIK NTM keratitis have increased in the literature(Chang, Jain et al. 2004).

Environmental investigation to identify the source of post-LASIK keratitis has been rarely reported and frequently fails to determine the origins and clonal relatedness of contaminating organisms with the causative agent. Some publications

suggest that the infection could be caused by the contaminated microkeratome, but this hypothesis has not been fully confirmed (Reviglio, Rodriguez et al. 1998; Garg, Bansal et al. 2001). In an outbreak of infections after LASIK caused by *M. chelonae* reported in Southern California, the masks created from a soft contact lens were used in hyperopic LASIK and were determined as the source of infection, but mycobacteria were not detected in additional lenses from the same lot. A thorough environmental investigation failed to identify the source of infection, but cultures were performed more than three weeks after recognition of the outbreak (Chandra, Torres et al. 2001).

The study of an outbreak of post-LASIK *M. chelonae* infections in São Paulo, Brazil, in 2000 revealed that samples from the air-conditioning system and water from the portable steamer contained the same strain of *M. chelonae* isolated from the patients, but the investigation was not specifically directed at finding possible sources of contamination (Freitas, Alvarenga et al. 2003).

In a subsequent outbreak that occurred in Brazil, an environmental investigation was carried out one month after an outbreak after LASIK caused by *Mycobacterium immunogenum* and affecting five patients. Samples of tap water, antiseptic and anesthetic solutions, nonsurgical soap, commercial distilled water, water from a bench-top autoclave and from the air-conditioning system drain were collected, decontaminated and cultivated on Middlebrook 7H10 agar. None of the environmental cultures yielded mycobacteria (Sampaio, Junior et al. 2006).

In one outbreak reported, a definite source was identified. The five confirmed cases were caused by *Mycobacterium szulgai* and the same species was isolated from the ice machine's drain. Isolates from patients and from the ice machine showed indistinguishable PFGE patterns, confirming the contamination from ice that was used to chill syringes of saline solution used for intraoperative lavage (Holmes, Bond et al. 2002).

There are multiple possible sources of keratitis including the patient's conjunctival and lid microbiota, the microkeratome or other surgical instruments and postoperative inoculation by the patient. Contaminated water is frequently considered as the most likely source, but rarely confirmed in outbreak reports. Failure to use appropriate aseptic techniques has been considered as a contributing factor (Chang, Jain et al. 2004). The use of the same microkeratome blade in both eyes operated

simultaneously is a common practice, but it is unclear whether the lack of blade change between eyes in a simultaneous bilateral LASIK surgery would increase the risks of infection. A case of *M. chelonae* bilateral keratitis has been documented in which the surgeon refers to having used the same set of instruments and the same microkeratome blade on both eyes, emphasizing the contamination by instruments during surgery in bilateral occurrences (Garg, Bansal et al. 2001). Others have suggested, by observing the tissue patterns of infection evolution on the corneal stroma in a patient that underwent LASIK, that *M. chelonae* contamination of the microkeratome was the cause the postoperative development of keratitis (Kouyoumdjian, Forstot et al. 2001).

NTM are present in different natural and artificial ecosystems as lakes, rivers, soil and potable water supply systems. Human beings are surrounded by these opportunistic pathogens (Falkinham 2009). Several publications have indicated that infections caused by NTM may originate from contaminated water (Conger, O'Connell et al. 2004; Brown-Elliott and Wallace Jr. 2012a). NTM not only survive but also multiply in water distribution systems (Falkinham 2002; Le Dantec, Duguet et al. 2002b). Le Dantec *et al*. demonstrated that *M. aurum* and *M. gordonae* isolated from water delivery systems are 100 and 330 times more resistant to chlorine than *Escherichia coli,* respectively (Le Dantec, Duguet et al. 2002a). The elimination of microorganisms after water chlorination reduces competition and favors growth of chlorine-resistant bacteria, as those belonging to genus *Mycobacterium* (Falkinham, Norton et al. 2001; Le Dantec, Duguet et al. 2002a). Biofilms containing NTM may play a role in resistance against disinfection and sterilization procedures and favor the occurrence of outbreaks like the one studied here (Falkinham 2013).

In this study, results of microbiological investigation of specimens from patients and from environmental samples, collected two months after the detection of the last case, confirmed that the strain of *M. chelonae* isolated from distilled water samples caused infections in four patients. The environmental and clinical isolates were genetically identical by DNA sequencing and by macrorestriction profile of whole cell DNA content examined by PFGE. Environmental and clinical isolates were highly resistant to the antimicrobial agents tested. Levels of resistance as determined by individual MICs for each drugs were similar, except for moxifloxacin for which the MIC value was 4-fold and 8-fold higher with patients' isolates than with the water

isolate. This is likely to be the result of previous exposure to fourth-generation fluoroquinolones which were administered prophylactically after surgery and which were also empirically administered when the patients presented with symptoms of infection. Interestingly, gatifloxacin MIC values of the two patients' isolates were high (>16 μg/ml), but all outbreak patients were treated with drops containing gatifloxacin and responded well, ending with 20/20 vision.

In the surgical facility evaluated in this study, tap water was distilled on a daily base in a bench distiller, stored in a non-sterile tank and used to rinse instruments and to fill the portable steamer and autoclave reservoirs. Therefore, tap water could have been the original source of this *M. chelonae* strain, which persisted in biofilms in the storage tank together with other biofilm-forming microorganisms, as the Gram-negative bacteria and yeasts that were isolated from the same distilled water samples. The investigation revealed the presence of *M. mucogenicum* in the tap water sample, indicating that NTM could be present in the water delivery system of the clinic.

However, the presence of potential infection agents in distilled water cannot explain all aspects of the occurrence of this outbreak. Infections caused by the other isolated organisms were never detected in this clinic. One hundred and seventy two surgeries were performed in this particular surgical facility during the cluster period. A total of 15 surgeons used the laser facility from December 2011 to February 2012. One surgeon operated 7 out of the 8 patients that developed infection. All those 7 infections were bilateral. Statistical analysis showed a statistically significant risk of developing mycobacterial infection when this particular surgeon was included in the analysis (data not shown). Therefore it is possible that technical differences among the surgeons may play an important role in the occurrence of post-LASIK infections. The only patient that was operated by a second surgeon was also the only patient that had unilateral infection.

In conclusion, the use of tap water distilled locally represents a great risk of infection in ophthalmologic surgical facilities and should never be used to rinse surgical instruments even through the steamer equipment. Special care should be taken to ensure that the water supply, air filters, and sterilizing facilities in the laser center are free of contamination to reduce the probability of outbreaks. Finally, optimization of technical aspects of the surgical procedure is of utmost importance.

ACKNOWLEDGMENTS

This work received financial support from Fundação de Amparo à Pesquisa do Estado de São Paulo (www.fapesp.br; FAPESP) (processes no. 2010/52641-1 and 2011/18326-4). CLN and CKM received fellowships from FAPESP (Processes no. 2012/13763-0 and 2013/16018-6).

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